Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 102 NOVEMBER 1959 No. 2

SECTION MEETINGS

DISTRICT OF COLUMBIA
George Washington University October 1, 1959
SOUTHERN
Tulane University September 24, 1959

Influence of Nicotinic Acid on Hepatic Cholesterol Synthesis in Rabbits.* (25214)

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The clinical use of nicotinic acid to reduce serum cholesterol has been widely accepted (1,2). Although there seems reasonable agreement concerning the efficacy of this drug in lowering cholesterol concentration, there is no agreement as to the biochemical mechanisms operative in the process. Altschul(3) implies that nicotinic acid is converted to either diphospho- or triphosphopyridine nucleotide, which in turn, enhances the oxidative metabolism of cholesterol. It is interesting, however, that nicotinamide, the active moiety of these coenzymes, is ineffective in decreasing cholesterol levels(4). Kraupp *et al.*(5,6) propose that nicotinic acid activates the reticuloendothelial system which in turn alters chemical distribution of the cholesterol in the blood. Another hypothesis has been advanced (7) in which the release of heparin from depot stores by nicotinic acid leads to a depression of serum cholesterol. Merrill(8) has presented experiments which indicate that nicotinic acid enhances the rate of acetate-1-C^{14} incorporation into cholesterol. His finding of a 70-90% increase in rate of sterol synthesis by rat liver, *in vivo,* would demand even greater catabolism of cholesterol in order to produce the lowered blood levels. Recently Schön(9) demonstrated that *in vivo* formation of total hepatic cholesterol is depressed proportionally to the amount of nicotinic acid fed to rats. This inhibition of synthesis was attributed to a lack of acetyl-Coenzyme A needed for cholesterol as well as fatty acid synthesis. Since the effect of nicotinic acid in reducing serum cholesterol in rabbits closely parallels the human response, we have investigated the rate of its synthesis in rabbit liver slices from animals under different dietary regimens, by measurement of both total liver cholesterol as well as rate of incorporation of acetate-1-C^{14} into this compound. It will be shown in this report that nicotinic acid significantly reduces the rate of biosynthesis of cholesterol and a tentative mechanism is proposed.

Methods. Eight male rabbits were divided into 4 groups and fed the diets indicated below for a period of 6 months. Group I—Al-

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* This research supported in part by grant from U.S.P.H.S. Facilities of the Allan Hancock Fn. were generously provided.

TABLE I. Effect of Dietary Nicotinic Acid and Cholesterol on Biogenesis of Cholesterol by Liver Slices.

Group	Diet	Total liver cholesterol (mg/g, wet wt)	Specific activity of digitonide (counts/min./mg)	Relative rate of cholesterol synthesis (counts/min./g liver)
I	Ration	11.8	440	5200
II	" + nicotinic acid	8.5	319	2710
III	" + cholesterol	34.6	93	3220
IV	" + cholesterol + nicotinic acid	31.2	66	2060

bers Rabbit Family Ration, Group II—idem. + .5% nicotinic acid, Group III—idem. + 2% cholesterol, Group IV—idem. + .5% nicotinic acid + 2% cholesterol. At the conclusion of the feeding period, the rabbits were sacrificed by decapitation, their liver extirpated, and immediately chilled in phosphate buffer, pH 7.4. A 20 g sample, wet weight, of each liver was homogenized in a Waring Blendor with 80 ml of a 30% alcoholic KOH solution (w/v), filtered, and brought to a final volume of 100 ml. Total liver cholesterol was determined on an 0.1 ml aliquot of each homogenate by the Henly modification of the Zlatkis procedure (10). The remaining portions of the livers were then sliced with a Stadie-Riggs hand microtome. Approximately 1 g of weighed slices was incubated at 37°C, with shaking in a phosphate buffer, pH 7.4, containing K_2HPO_4 0.067 *M*; KH_2PO_4, 0.042 *M*; $MgCl_2$, 0.006 *M;* and acetate-1-C^{14} (specific activity approximately 1 mc/mM, 4 x 10^5 counts/min/ml) in a final volume of 5.0 ml. After 3 hours the slices and the medium were treated with 10 ml of sodium ethylate and brought to 85°C for 1 hour to saponify the lipid material. The non-saponifiable lipids were extracted 4 times with chloroform and the combined extracts taken to dryness with a stream of air. The residues were dissolved in 5 ml of a warm ethanol-acetone (1:1) solution and precipitated with a 1% digitonin in 80% ethanol solution. The digitonin precipitable fraction was removed by centrifugation, and resuspended in fresh ethanol-acetone solution. Aliquots were pipetted directly onto tared aluminum planchettes. The planchettes were dried in a stream of warm air, reweighed, and weight of the digitonide precipitate calculated. These samples were counted at infinite thinness in a gas flow counter.

Results. The data for total liver cholesterol as well as rate of incorporation of acetate-1-C^{14} into the digitonide precipitate are presented in Table I. Values for the relative rates of acetate incorporation into cholesterol per gram of liver are calculated from the product of the total cholesterol and specific activity of the digitonide in each case. It is evident, as often reported(11), that there is marked inhibition of cholesterol synthesis by exogenous dietary cholesterol. Further, there is a marked depression of cholesterol synthesis by dietary nicotinic acid both in the control and cholesterol fed animals. This latter finding is contrary to that of Merrill(8) who reported enhanced cholesterol synthesis by rat liver slices from animals fed nicotinic acid. Our data are, however, in agreement with those of Schön and permit a tentative explanation for the observed effect of nicotinic acid in lowering of serum cholesterol levels.

There is now considerable evidence to support a biochemical mechanism to account for the effect of nicotinic acid in lowering serum cholesterol. Reddi and Kodicek(12) using humans have demonstrated that the principal excretion product of intravenously injected nicotinic acid is nicotinuric acid. Miller(13) using hypercholesteremic patients and administering nicotinic acid orally found 20-25% of a 2 g/100 lb body weight dose was excreted as nicotinuric acid. The synthesis of nicotinuric acid has been described(14,15) as follows: nicotinic acid + glycine $\xrightarrow[\text{ATP}]{\text{Co A}}$ nicotinuric acid. Synthesis of cholesterol from acetate *via* mevalonic acid and the other attendant reactions(16) is intimately linked to participation of Coenzyme A (Co A). Thus nicotinic acid could act by effectively competing with the cholesterol synthesizing system for a small pool of free Co A. Nicotinamide is excreted as N^1-methyl nicotinamide, which is formed by reactions not requiring Co A. This may explain the inactivity of orally administered nicotinamide in reducing serum cholesterol. Confirmation for a hypothesis involving competition for Co A is presented by Wagner-Jauregg(17) who demonstrated that acetylation of sulfanilamide by a pigeon liver extract is inhibited by nicotinic acid and other compounds requiring Co A for detoxication. Schön's(9) demonstration of graded inhibition of cholesterol and total lipid synthesis in rats by increasing doses of nicotinic acid indicates diminished lipogenesis from acetyl-Co A. α-Phenylbutyric as well as diphenylbutyric acid, compounds requiring Co A in detoxication reactions, also effectively blocked lipid synthesis. Steinberg and Fredrickson (18) have also demonstrated inhibition of cholesterol synthesis from acetate-1-C^{14} *in vitro,* but failed to correlate their results with a competition for Co A.

Summary. 1) The mechanism by which ingestion of large amounts of nicotinic acid lowers serum cholesterol in rabbits has been investigated. The rate at which cholesterol is synthesized from acetate-1-C^{14} by liver slices from animals on control or cholesterol supplemented diets, with or without nicotinic acid, has been measured. 2) There is marked inhibition of the rate of cholesterol synthesis by liver slices from animals fed nicotinic acid on both control and supplemented diets. Since the principal detoxication product of large doses of nicotinic acid is nicotinuric acid, it is possible that this inhibition of cholesterol synthesis occurs as a direct result of competition of lipid synthesizing and detoxication systems for a limiting amount of Co A in the liver cell.

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Received July 31, 1959. P.S.E.B.M., 1959, v102.

Effect of 2,4-Dinitrophenol on Replication of Poliovirus in HeLa Cells.* (25215)

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The presence of critical concentrations of 2,4-dinitrophenol (DNP) in aerobic oxidative phosphorylative systems decreases the ratio of high energy phosphate atoms formed to the atoms of oxygen consumed and stimulates rate of oxidation of substrate. Thus, oxidation is permitted to proceed without the concomitant fixation of phosphorous. Replication of influenza virus(1-3), Theiler's GD VII mouse encephalomyelitis virus(4), and pneumonitis virus(5) has been reported to be inhibited by concentrations of DNP which increased respiration and substrate utilization of host cells but which decreased incorporation of inorganic phosphorous. In addition, synthesis of these same viruses were inhibited when host cells were maintained anaerobically (4-7). It has been postulated that the energy required for viral synthesis is supplied by high energy phosphate bonds generated by aerobic oxidation of substrate(1,5,6). On the other hand, it has been reported that poliovirus replicated in HeLa cells maintained anaerobically(8). In those studies the latent period of virus synthesis in cells maintained anaerobically was considerably prolonged but the final virus yield/destroyed cell was essentially the same as under aerobic conditions. The increased latent period can be interpreted as interference with the energy producing mechanisms required for virus synthesis. Since DNP also interferes with energy production it was of interest to investigate its effect upon virus synthesis using the poliovirus-HeLa cell system. Presumably, DNP should not inhibit replication of poliovirus in this system but should only prolong the time taken for synthesis if its mechanism of action is to uncouple phosphorylation solely from aerobic oxidative processes.

Methods. Poliovirus, type 1 (Mahoney strain) was employed as a pool of supernatant fluid from HeLa cell cultures of the 73rd virus passage. Virus suspensions were stored in ampules at –50°C. HeLa cells were grown according to the method of Syverton and Scherer(9). In place of human serum, serum protein prepared from outdated human blood bank blood was employed and supplemented with 0.2% yeast extract (Difco "Yeastolate"). For virus assay by the plaque technic, cells were trypsinized, dispensed, and established as monolayers in a medium consisting of 20% calf serum (CaS_{20}) or chicken serum (ChS_{20}) and 80% maintenance solution (MS_{80})(10) containing 0.2% yeast extract. The same medium was employed in manometric studies except that the yeast extract was omitted and 0.01 M tris(hydroxymethyl) aminomethane (THAM) was added as a supplementary buffer. *Manometric method.* Oxygen uptake was measured by the direct method in the conventional Warburg respirometer as previously described (11). Duplicate flasks were employed for each analysis. Krebs' modification of Pardee's carbon dioxide buffer was employed in oxygen uptake studies to maintain an equilibrium concentration of 2% carbon dioxide in the gas phase. *Virus assay.* Virus assays were done by the plaque method using HeLa cell monolayers in 30 x 60 mm screw-capped square, soft glass bottles(8). *Glucose.* Glucose was measured by a modified procedure using Dreywood's anthrone reagent(12). Cooled anthrone reagent, 4.0 ml, was added slowly to the samples in the icebath and they were shaken without removal. Such treatment resulted in little or no color development. Tubes were then placed into a boiling water bath for exactly 8 minutes after which they were rapidly cooled to room temperature. Optical density was read in the Beckman DU Spectrophotometer at 625 mμ. Concentrations of glucose of from 1 to 40 μg could be accurately estimated by this procedure. *Phosphorous.* Phosphorous was determined by the

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* Aided by grant from Nat. Inst. Health.

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TABLE I. Effect of 2,4-Dinitrophenol upon Glucose Utilization and Viral Replicating Capacity of HeLa Cells.

Conc. of 2,4-dinitrophenol ($\times 10^{-4}$M)	Glucose utilized, μg Infected	Control	Yield of poliovirus, PFU ($\times 10^5$)*
.0	102	112	12
1.0	135	162	16
2.5	133	202	19
10.0	0	0	0†

* Inoculum contained 300 PFU.

† No plaques on 10^{-4} dilution.

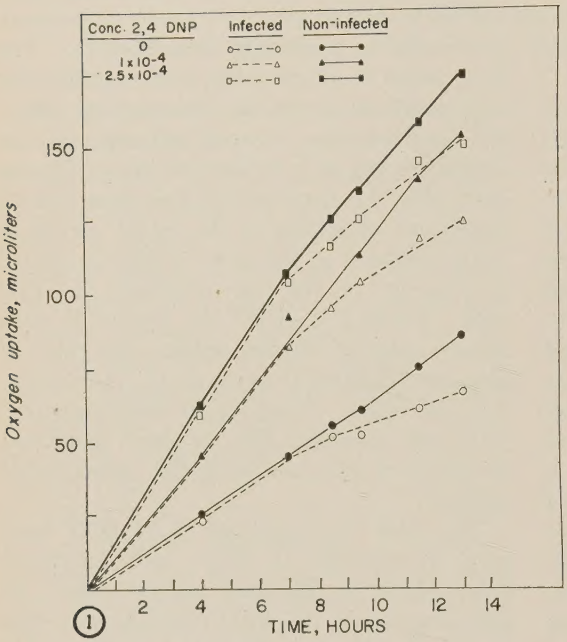
method of Fiske and Subbarow(13) or the modified procedure of Dryer, Tammes and Routh(14).

Results. The initial studies were performed with test tube cultures of HeLa cells. Concentrations of 2,4-dinitrophenol of 1 x 10^{-3} M, or greater, did not usually permit survival of HeLa cells. Concentrations of 1 and 2.5 x 10^{-4} M DNP permitted survival provided a well buffered medium was employed to prevent cell damage due to stimulation of glycolysis and concomitant acid production. Table I presents the results of an experiment in which approximately 300 PFU (plaque forming units) of poliovirus were added to replicate cultures of HeLa cells with various concentrations of DNP and incubated for 96 hours. Virus and DNP were added at the same time. Each HeLa cell culture contained approximately 80,000 cells. The results represent supernatant fluids from 5 replicate tubes and indicate that concentrations of 2,4-dinitrophenol which stimulated glycolysis did not inhibit replication of poliovirus. The data also show that the virus yield per cell was not adversely altered by these concentrations of DNP since the titers of virus given represent the total amount of virus in tubes after complete destruction of the cells.

The following procedure was done to test the possibility that the latent period of poliovirus replication in HeLa cells was altered by the presence of DNP. Warburg flask cultures containing approximately 1 x 10^6 freshly trypsinized HeLa cells were prepared and various concentrations of DNP added to flasks in quadruplicate. Two flasks of each concentration of DNP were inoculated with 1.5 x 10^6 PFU of poliovirus and the 2 remaining flasks inoculated with a similar amount of medium to serve as non-infected controls. This amount of virus would be sufficient to destroy a significant portion of the HeLa cells after one cycle of replication to be detectable manometrically as previously described(8). The time taken to destroy the virus synthesizing cells could be accurately measured by determining when these infected cell cultures consumed oxygen at a diminished rate. The results (Fig. 1), demonstrate that there was no significant alteration in the latent period of virus synthesis in the case of cells incubated in presence of the concentrations of DNP used. Table II includes the results of virus assays, rates of oxygen uptake and rates of glucose utilization in presence of 2 concentrations of DNP. Phosphorous determinations, total and inorganic, did not show significant deviation in any case from the uninoculated medium.

The same concentrations of 2,4 DNP were used in cultures of HeLa cells incubated anaerobically in Warburg respirometers. In this case, 1.6 x 10^7 PFU of poliovirus were employed with approximately 1 x 10^6 freshly trypsinized HeLa cells. The results are shown in Fig. 2. There was no apparent effect of these concentrations of DNP upon rate of acid production by HeLa cells under anaerobic conditions as indicated by rate of release of carbon dioxide from the bicarbonate buffer. Similarly, there was no alteration in the course of carbon dioxide evolution from infected cultures in the presence or absence of DNP. The delay in virus replication under anaerobic conditions, as previously determined by this procedure(8), was apparent and was not altered by the presence of DNP.

Discussion. Our results presented validate the hypothesis, based on results of anaerobic studies with HeLa cell and poliovirus(8), that poliovirus would be replicated in HeLa cells treated with critical concentrations of 2,4-dinitrophenol which markedly stimulated oxygen uptake and glucose utilization under aerobic conditions. Similar virus titers were obtained with concentrations of DNP which stimulated respiration as with the control cultures. Concentrations of DNP which inhibited glycolysis also inhibited virus synthesis; however, these concentrations of DNP did not



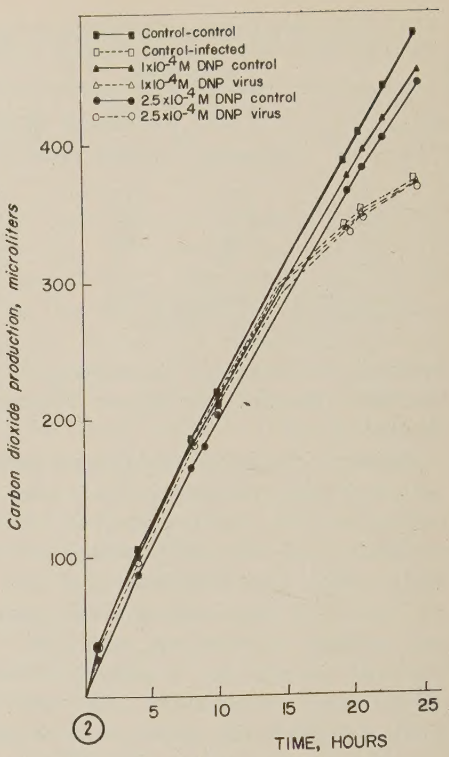


FIG. 1. Effect of 2,4-dinitrophenol upon respiration of infected and non-infected HeLa cells.

FIG. 2. Effect of 2,4-dinitrophenol upon release of carbon dioxide from bicarbonate buffer by HeLa cells under anaerobic conditions.

appear to be compatible with survival of the cells since the latter did not remain attached to the glass.

Unlike the anaerobic effect, 2,4-dinitrophenol did not alter the latent period of virus synthesis. The time required before a decrease in rate of respiration of infected cells became apparent was similar in the presence or absence of the agent. These results suggest that the energy obtained through anaerobic glycolytic pathways by HeLa cells was sufficient to permit replication of poliovirus and hence it is possible that the prolonged latent period found under anaerobic conditions (8) was not due to a lack of energy but rather to another unrecognized factor.

The failure to find any significant difference in inorganic or organic phosphorous in the medium before and after exposure of the cells to this medium demonstrates that the medium is a true maintenance medium since net growth should result in phosphate uptake. Since the inorganic and organic phosphorous concentrations of the medium remained constant in the presence of a marked increase in rate of oxygen uptake in the presence of DNP there was an uncoupling of oxidation and phosphorylation. Utilization of glucose was

TABLE II. Effect of 2,4-Dinitrophenol upon Oxygen Uptake, Glucose Utilization and Virus Yield over a 21 Hour Incubation Period.

Conc. of 2,4-dinitrophenol ($\times 10^{-4}$M)	O_2 uptake, μl/hr	Glucose utilized, μg/hr	O_2 uptake (moles) / Glucose used (moles)	Virus yield, PFU ($\times 10^7$)
.0	6.5	10.2	5.02	4.6
1.0	12.0	66.3	1.28	4.3
2.5	14.0	104.8	.95	1.7

more greatly stimulated than oxygen uptake by the presence of DNP as shown by the molar ratios of the 2 substances. Apparently the dissociating effect of DNP on aerobic phosphorylating systems in HeLa cells is compensated by greatly increased glycolysis and anaerobic phosphorylating systems. An increase of glucose uptake of 10-fold was found with 2.5 x 10^{-4} M DNP whereas uptake of oxygen was increased only 2-fold. These observations may explain the apparent discrepancies between the results presented here and those previously reported(1-7) since it is possible that the tissue cells they employed could not compensate as well with non-aerobic phosphorylating systems under conditions of anaerobiasis or in the presence of DNP. Furthermore, these previous reports on inhibition of virus synthesis by DNP have all used cells which were derived from normal animals whereas HeLa was derived from malignant tissue. Warburg(15) has stated that in cancer cells there is an increase in fermentation such that the failure of respiration is compensated for energetically.

Anaerobically maintained HeLa cells did not appear to be affected by the concentrations of DNP which markedly alter the metabolism of aerobically maintained cells. This is in accord with the generally accepted view that this substance interferes solely with aerobic processes.

Summary. Poliovirus was found to replicate in HeLa cells treated with critical concentrations of 2,4-dinitrophenol which markedly stimulated oxygen uptake and glucose utilization under aerobic conditions. The time required before a change in rate of respiration of infected cells became apparent was similar in absence or presence of DNP, indicating that this substance did not alter the latent period of virus synthesis. Apparently the uncoupling effect on aerobic phosphorylating systems is compensated for by increased glycolysis and the concomitant fixation of phosphorous by systems not effected by DNP.

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Received July 31, 1959. P.S.E.B.M., 1959, v102.

Fat Mobilization by Purified Corticotropin in the Mouse.*† (25216)

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Purified corticotropin (ACTH) is known to produce acutely fatty liver and ketonemia in rats and mice(1). These signs of accelerated fat catabolism appear not to result from stimulation of the adrenal cortex but to reflect a direct action of ACTH on intermediary fat metabolism of extra adrenal tissues(2). Evidence is available that the similar activity of growth hormone (STH) is not due to mutual contamination but represents another of many effects common to the two hormones (1,2). The suggestion has been made that both ACTH and STH accelerate fat catabolism by stimulating fat mobilization from peripheral depots(3,4). In support of this viewpoint are their lipolytic effects on rat adipose tissue *in vitro*(5). Growth hormone has considerably less and more variable lipolytic activity *in vitro* relative to ACTH however than would be expected if this direct action of each hormone on adipose tissue were the sole source of their more nearly equal ketogenic potencies in intact animals. Data presented here provide further evidence *in vivo* that ACTH and STH do speed the loss of stored lipid from depots of fasted mice and that this action of ACTH is independent of adrenal stimulation.

Materials and methods. Swiss mice weighing 18-21 g were fasted overnight, anesthetized with ether, and one testis, alternately right or left, removed from each through a scrotal incision. The epididymal fat pad was then carefully dissected away, hormones were given intraperitoneally in a volume of 0.1 ml, and the mouse allowed to recover from anesthesia. It was maintained on tap water for 6 hours, then reanesthetized and the second fat pad and liver removed. Total lipids were determined on fat pads and liver by the method of Handler(6). The difference in total extractable lipids between the two pads was taken as a measure of fat mobilization, expressed in Table I both in mg and as per cent of total lipids in the initial pad. One group of mice was adrenalectomized through dorsal incisions at the time the first fat pad was removed and just prior to treatment with hormones. This group was maintained for the subsequent 6 hours on 0.9% saline. Preliminary studies demonstrated no significant difference in lipid content of the 2 pads of the same mouse when they were removed together, as Stoerck and Porter have found in the rat (7). The hormones used were hydrocortisone as the hemisuccinate, The Upjohn Co.; oxycel adsorbed ACTH, Wilson Laboratories, lot No. 102621, approximately 140 U.S.P. units per mg; growth hormone, Endocrinology Study Section, Nat. Inst. of Health, BGH-1.

Results. No mobilization of lipid was detected in the untreated controls during the 6 hour period of observation, as shown in the upper section of Table I. Growth hormone and ACTH, however, both caused significant losses of depot lipid. A small or large dose of hydrocortisone failed to effect similar lipid depletion, indicating that neither ACTH nor STH was acting through stimulation of the adrenal cortex. The liver fat values of these intact mice were not significantly different. Previous studies(1) have demonstrated that female mice of this same strain develop fatty livers within 4 hours of receiving ACTH or STH intraperitoneally. Average values for liver fat in this study were highest for the groups treated with growth hormone or corticotropin and lowest in the groups given hydrocortisone. Failure to achieve significance in these differences may relate to the use of male animals, which appear to be more resis-

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* Supported by grants from Am. Cancer Soc., Nat. Inst. of Arthritis and Metab. Dis. and by Office of Surgeon General, Dept. of Army.

† We are indebted to Drs. C. J. O'Donovan, Upjohn Co., Kalamazoo, Mich., for hydrocortisone hemisuccinate, David Klein, Wilson Labs., Chicago, Ill., for oxycel corticotropin, and to Endocrinology Study Section of Nat. Inst. of Health for bovine growth hormone.

‡ Amer. Diabetes Assn. Fellow, 1957-59.

TABLE I. Lipid Mobilization from Epididymal Fat Pads of Fasting Mice.

Treatment	No. animals	Avg body wt (mg)	Epididymal lipid (mg)				mg liver fat per 100 mg liver
			Initial	Final	Difference	% difference	
			Intact				
None	15	18.2	57.1	56.4	– .8 ± .99	– 2.5 ± 1.94	5.45 ± .321
STH .5 mg	12	17.8	63.8	54.6	*–9.3 ± 1.17*	*–17.3 ± 2.54*	6.13 ± .454
ACTH .1	8	19.4	48.7	40.3	*–8.4 ± 1.28*	*–20.8 ± 4.80*	5.96 ± .542
HC .1	9	20.1	60.3	57.2	–3.0 ± 3.10	– 5.1 ± 5.32	5.01 ± .239
HC 1.0	8	18.2	59.5	57.7	–1.8 ± 1.25	– 4.1 ± 2.34	4.82 ± .121
			Adrenalectomized				
None	22	20.7	82.0	75.8	–6.2 ± 1.69	– 7.9 ± 1.73	5.11 ± .243
p					<.05	<.05	
HC .1 mg	9	21.9	88.6	86.7	–1.9 ± 1.17*	– 2.6 ± 1.47‡	4.34 ± .340*
ACTH .1	23	20.7	95.7	87.9	–8.2 ± 1.94†	– 8.1 ± 1.58§	6.43 ± .316†
HC .1 mg + ACTH .1 mg	21	20.7	93.4	84.8	–8.7 ± 2.13†	– 9.6 ± 2.06§	6.23 ± .291†

Values are means ± stand. errors. Those in *italics* are significantly different (p <.01) from control value at top of respective column.

† p <.01 over *. § p <.02 over ‡. p <.05 shown between means compared. STH—growth hormone. ACTH—corticotropin. HC—hydrocortisone as the hemisuccinate.

tant to fatty liver than the female. Under the conditions of this study loss of depot fat in the intact mice appears to be a better indicator of fat mobilization than development of fatty liver.

Further evidence that ACTH stimulates fat mobilization by means other than adrenal stimulation may be seen in the lower section of Table I. Mice adrenalectomized after removal of the first fat pad lose significantly more lipid from the remaining pad in 6 hours than do mice with intact adrenals. The controls necessary to identify this increased fat mobilization with the adrenal deficit rather than with the more extensive surgery were not done. Levy and Ramey(8), however, have shown in the fasted rat that hydrocortisone administration or sham adrenalectomy inhibits mobilization of depot fat, whereas adrenalectomy promotes it. Furthermore, in the present study hydrocortisone inhibited fat loss from depots of adrenalectomized mice. ACTH failed to accelerate fat loss over the effect of adrenalectomy alone (Table I) due perhaps to a pre-existing maximal stimulus to fat mobilization. ACTH given to adrenalectomized mice together with hydrocortisone, however, overcame the inhibition of fat depletion which occurred with this dose of hydrocortisone alone. Further evidence of fat mobilization by ACTH in adrenalectomized mice is the significantly higher values for liver fat in the animals treated with ACTH or ACTH plus hydrocortisone than those treated with hydrocortisone alone.

Discussion. The data presented here serve as a link between earlier studies demonstrating (a) ketosis and fatty liver following ACTH and STH administration *in vivo* and (b) acceleration of lipolysis in isolated adipose tissue exposed directly to the same hormones *in vitro*. Viewed as a whole the evidence strongly favors the interpretation that both hormones may act directly on adipose tissue depots to promote lipid mobilization. The scant but significant lipolytic effect of STH on adipose tissue *in vitro* relative to ACTH leaves open the possibility, however, that growth hormone may deplete fat depots *in vivo* by more than this one means. The relative importance to fat mobilization of endogenously secreted hormones, including epinephrine and norepinephrine, still is uncertain. The results of this study are nevertheless consistent with the possibility that increased ACTH secretion in response to adrenalectomy contributed to the greater fat loss from depots of the adrenalectomized mice than occurred when the adrenals were left intact. Hydrocortisone has not been found to inhibit lipolysis when applied directly to adipose tissue *in vitro*(5). It seems possible that it may inhibit fat mobilization in the adrenalectomized mouse by opposing the secretion of endogenous ACTH. Alternative explanations have not been excluded, however, such as stimulation by hydrocortisone of insulin secretion from the pancreas. Further suggestive evidence that endogenously secreted pituitary hormones may effect fat mobilization is the well known tendency of hypophysectomized rats to become relatively obese when fed and to resist lipid depletion when fasted despite their proneness to hypoglycemia. Knobil(9) has demonstrated that adipose tissue from hypophysectomized rats releases smaller amounts of fatty acid *in vitro* than that from normal rats, whereas treatment of the hypophysectomized rats with STH restores this defect toward normal. The contributions of increased insulin sensitivity and lowered basal metabolic rate to impaired fat mobilization of such animals are yet to be evaluated.

Summary. Purified corticotropin and growth hormone both stimulate lipid mobilization from the epididymal fat depots of fasting mice. Hydrocortisone failed to induce lipid loss in intact mice and inhibited fat mobilization in adrenalectomized mice, indicating that the lipid mobilizing effect of corticotropin is not mediated by adrenal stimulation. Furthermore, inhibition of fat loss by hydrocortisone in adrenalectomized mice could be overcome by simultaneous administration of corticotropin. The results demonstrate an extra-adrenal action of corticotropin on fat mobilization in the intact animal similar to that induced by growth hormone.

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Received July 27, 1959. P.S.E.B.M., 1959, v102.

Mechanism of Inhibition of Gastric Secretion by Fat in the Intestine.*

(25217)

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It is well known that gastric secretory activity and gastric motility are inhibited when fat is present in the proximal small bowel(1). This inhibitory effect of fat has been documented by experiments in dogs and humans, and is said to be mediated by an agent circulating in blood(2). The nature of this agent is unknown. Lim *et al.*(3) prepared from mucosa of small and large bowel of dogs a crude extract capable of inhibiting gastric secretion in dogs. They postulated that these extracts and also those prepared later from hog small bowel mucosa contained the hormone "enterogastrone" which under physiologic conditions is released by the small bowel mucosa in the presence of fat. Whereas "enterogastrone" preparations inhibit gastric secretion in dogs and to a lesser degree in humans, they fail to inhibit gastric secretion in the rat(4). Our finding(5) that parenteral administration of bile salts inhibited gastric secretion in rats suggested the possibility of increased resorption of bile salts incident to fat digestion and absorption being the mechanism responsible for gastric inhibition by fat. Our purpose was to determine if rat gastric secretion was inhibited when fat was present in the small intestine and, if so, to what extent this inhibition was affected by presence

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* The author acknowledges the technical assistance of Howard Guiles.

† Markle Scholar in Medical Sciences.

TABLE I. Influence of Presence or Absence of Bile Salts in Lumen of Small Bowel on Inhibition of Gastric Secretion of Pylorus Ligated Rats by Fat in the Intestine.

		Mean % change from paired controls	
	No. rats†	Vol (ml)	Free HCl (meq/l)
Intact rats, 2 ml olive oil*	30	–80% $p < .001$	–35% $p < .001$
Bile shunt, ” ” ” *	38	–16% p .1	+ 1%
Bile shunt, ” ” ” + 400 mg Tween 80*	26	–10% $p < .2$	+ 1%
Bile shunt, ” ” ” + 30 mg ox bile salts*	20	–81% $p < .001$	–43% $p < .001$
Bile shunt, 30 mg ox bile salts*	16	–3%	+13%

* Intraduodenal administration.
† Total No. of paired control and treated rats in each group.

or absence of bile from the intestine. Our data show that gastric secretion also in the rat is inhibited by fat. This takes place only when bile constituents, particularly bile salts, as well as fat are present in the small bowel.

Materials and methods. Gastric secretion was measured by pyloric ligation method(6) in 130 rats weighing 160 to 220 g. Prior to pyloric ligation, the rats were fasted for 48 hours in individual cages with wide mesh wire bottoms. During fasting period water was given *ad lib.* Rats were divided into 5 groups of paired control and treated rats. In all rats *except those of Group 1,* the bile duct was shunted to the rectum 24 hours before pyloric ligation, using a technic described previously (5). The entire bile output is diverted from the small intestine and coecum. Pancreatic secretions are left undisturbed. It was established previously that diversion of bile from the small intestine does not alter gastric secretion in the rat. In rats belonging to Group 1 a sham operation was done. Pyloric ligation was performed in all rats under light ether anesthesia. Simultaneously, treated rats received the following intraduodenal injections. Groups 1 and 2: 2 ml of olive oil each. Group 3: 2 ml of olive oil and 400 mg of Tween 80. Group 4: 2 ml of olive oil and 30 mg of ox bile salts. Group 5: 30 mg of ox bile salts in 2 ml of normal saline. Doses were on basis of 200 g of rat weight. They were administered by single injection through a 26 gauge needle inserted into the duodenum immediately distal to pyloric ligature. The paired random controls received the same volume of normal saline. Six hours later the rats were killed and stomachs removed. The gastric content was studied individually for volume and free HCl concentration.

Results. The data are summarized in Table I. Intraduodenal administration of olive oil suppressed gastric secretion in intact rats. Only moderate inhibition of gastric secretion was observed when the same amount of oil was administered to rats of Group 2 in which bile had been diverted from small intestine. This result was not altered by addition of Tween 80 to the oil. However, the same degree of suppression of gastric secretion observed in intact rats occurred when bile salts were added to the oil. Finally, administration of bile salts alone into the duodenum did not alter gastric secretory activity.

Discussion. These data show that the presence of fat in the small intestine decreases gastric secretory activity in rats (as well as in dogs and humans). Following diversion of bile from the small intestine, gastric secretion is not inhibited by fat unless bile salts are added to the administered oil. On the other hand, this same dose of bile salts which had been shown to suppress gastric secretion when administered parenterally, did not alter gastric secretion when given intraduodenally in the absence of fat. It seems unlikely that the relationship of bile salts to gastric inhibition by fat is nonspecific and due merely to their surface active properties in view of the results obtained in Group 3 in which addition of

Tween 80 to the oil did not result in any greater inhibition than observed in Group 2. There is some experimental evidence to suggest that fatty acids are more potent inhibitors of gastric motility than neutral fats(7). Therefore, one could postulate that the lack of inhibition of gastric secretion by olive oil when bile was absent from the small bowel was due to a lower concentration of products of hydrolysis of neutral fat in the intestinal lumen. However, percentage hydrolysis of neutral fat is often normal in spite of absence of bile salts(8). Furthermore, oleic acid, when administered intraduodenally to intact rats, does not have as strong and as consistent inhibitory effects on gastric secretion as olive oil (unpublished data).

Summary. Gastric secretion in the intact rat is inhibited by fat in the small intestine. This inhibitory activity is decreased when bile has been diverted previously from the small intestine. Under these same conditions, addition of bile salts to administered fat results in the same degree of inhibition as in the intact animal.

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Received June 22, 1959. P.S.E.B.M., 1959, v102.

Comparative Plasma Levels of Mephenesin, Mephenesin Carbamate and Methocarbamol.* (25218)

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It has been shown in both animal and man, that significantly higher and more persistent plasma levels were obtained by mephenesin carbamate as compared to those of mephenesin(1,2). When a similar pair of related drugs, namely glyceryl quaiacol ether (G.G.E.) and its carbamate, methocarbamol, were compared by several methods, it was found that methocarbamol gave higher plasma levels than G.G.E. Furthermore, methocarbamol gave higher plasma levels than mephenesin or mephenesin carbamate, following oral administration of equimolar amounts of the drugs to dogs(1). Since similar observations in man have not been reported, it was decided to determine the plasma levels of mephenesin, mephenesin carbamate and methocarbamol, following oral administration of equimolar doses of these drugs to humans. Such studies should be of interest because of clinical reports on the prolonged action of methocarbamol in the treatment of painful muscle spasm(3,4,5,6,7).

Materials and methods. Ten healthy subjects, six males and four females, were chosen. Four subjects (Group 1) were started on methocarbamol, three (Group 2) on mephenesin, and three (Group 3) on mephenesin carbamate. About one week later, when the blood was free of these drugs the subjects of the three groups ingested mephenesin or mephenesin carbamate, or methocarbamol in such a manner as to give randomness in the sequence of ingestion of the drugs. The dosage was 4.00 g of methocarbamol; or 3.75 g of mephenesin carbamate; or 3.00 g of mephenesin. These amounts of drugs are equimolar. The total dose was ingested at once—immediately

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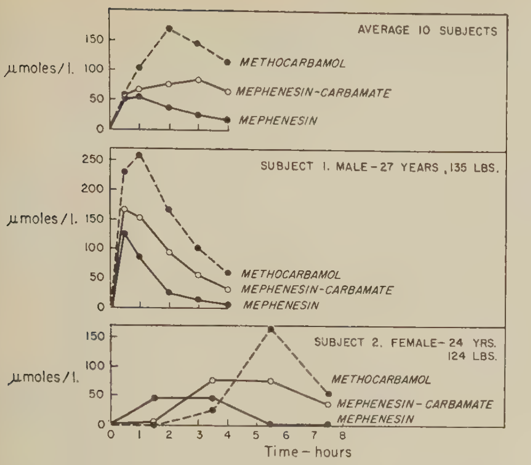
* Supported by grant from A. H. Robins Co., Inc., Richmond, Va.



after a control blood sample was obtained. Further blood samples were drawn one-half, one, 2, 3 and 4 hours following the medication. In one subject the studies were repeated and several blood samples were drawn over a period of 7½ hours. After deproteinizing the serum with Folin-Wu reagent, the drugs were extracted with chloroform from an aliquot of the filtrate. The further analytical procedure involved the coupling with a diazonium compound and colorimetric estimation of the complex. In all details we followed the method described by Titus *et al.*(8).

Results. The results of these studies are shown in the accompanying graph. Plasma levels are plotted on the ordinate and are expressed in micromoles per liter of blood because the drugs were administered in equimolar amounts. For comparison on a milligram percent basis, 100 micromoles per liter are equivalent to 2.41 mg% methocarbamol, 2.25 mg% mephenesin carbamate, and 1.82 mg% mephenesin, respectively. In agreement with the observations by London and Poet, it was found that mephenesin carbamate gave higher peak values than mephenesin. Methocarbamol exceeded both drugs in this regard. When the average data (upper part of illustration) were treated statistically, it was found that the differences between methocarbamol on one side and mephenesin and mephenesin carbamate on the other side were highly significant ($P<0.01$), with the exception of the values for the one-half hour levels. As might have been expected, individual differences in the time course of the blood levels were noted. Most subjects gave results as shown in the middle section of the illustration, which are the data of Subject No. 1. Subject No. 2 reacted differently from the others as shown in the lower section of the illustration. When the observation time was extended from 4 to 7½ hours, however, this subject, too, showed that mephenesin carbamate reached higher plasma levels than mephenesin and methocarbamol higher levels than mephenesin or its carbamate.

It is impossible at the present time to interpret the plasma levels of the drugs studied in terms of rates of absorption, excretion and metabolism of the drugs, because of lack of sufficient information concerning these points.

Summary. Ten normal subjects ingested equimolar amounts of mephenesin, mephenesin carbamate and methocarbamol, with intervals of at least one week between medications. Blood levels of drugs were followed for 4 hours, and in one case for 7½ hours. In all subjects mephenesin carbamate gave higher peak values than mephenesin. Methocarbamol exceeded the other 2 drugs significantly in this regard.

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Received January 21, 1959. P.S.E.B.M., 1959, v102.

Test Based on Normal Serum Component Implementing Fixation of Complement by Turkey Antiserum.*† (25219)

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Because heated turkey and chicken antiserum failed to fix complement in the presence of homologous antigen, Bushnell and Hudson (1) and later Rice(2) reported a direct complement fixation test on unheated fowl serum. However, they discarded this method because of anticomplementary quality of avian serum. In a direct complement fixation (CF) test for ornithosis antibody in turkey serum, Benedict and McFarland(3) were successful in using heated serum with a purified detergent extracted antigen or fresh unheated serum with the anticomplementary effect removed by adsorption of serum with kaolin. Brumfield and Pomeroy(4) first developed a direct test that used sufficient complement to overcome the anticomplementary effect. After publication of this method it was found that titers could be increased 2 to 8 fold in many sera by addition of fresh normal chicken (or turkey) serum to heated or unheated serum antibody. The antigen was not purified nor was the use of this normal labile factor restricted to the ornithosis system. Although the greater part of this work was performed with turkey antiserum and crude yolk sac ornithosis antigen, pilot experiments showed the principle applied to bacterial and viral systems where antigens were made in a variety of ways.

Materials and methods. Test for ornithosis antibody in turkey serum. 1) Veronal buffered saline, pH 7.3 containing 0.00015 M $CaCl_2$ and 0.0005 M $MgCl_2$ was used for diluting all reagents. 2) The suspension of sheep erythrocytes was stored at 4°C in Alsever's solution. Cells were washed at least 3 times or until supernate was clear, made up to 4% suspension and sensitized with equal volume of hemolysin containing 2 units hemolysin/ml. Final suspension 2% sensitized with 2 units hemolysin. 3) Bacto-Antisheep Hemolysin. Difco Lab. 4) Frozen guinea pig serum pre-tested for lack of reaction with psittacosis antigen. For each new pool of complement, dilution was determined by incubating 0.1 ml (half portion) at dilutions 1/20, 1/22, 1/24, with 0.2 ml of 10 different unheated turkey sera diluted 1/8 and 0.3 ml diluent. The hemolytic system was added after 1 hour (0.2 ml). Readings were made 30 minutes later. The highest dilution of complement in 0.1 ml (usually 2 units) causing hemolysis of indicator system was considered half portion. This amount was used in both serum and antigen controls. Twice that amount (usually 4 units) was used in the test. 5) More than 2 units of antigen, preferably 4 units was used. Antigen was titrated with 4 units of unheated fresh or frozen turkey serum. Antiserum of other species seldom gave the same titer. 6) For enhancement and stabilization of the test, normal chicken (N-ch) serum was incorporated in the system. N-ch should be from a pool. It was collected and stored as quickly as possible at –20°C to preserve the labile substance. This serum was tested for lack of reaction with antigen. 7) Usually test sera were not heated, they were tested fresh or after storage at –20°C. The order of addition of reagents was *mandatory.* (a) First, 0.2 ml of test serum dilution was dispensed, (b) followed by approximately 0.01 ml N-ch delivered from a 0.2 ml pipette. Because pools of N-ch vary, amount of N-ch was determined by following titration. From one set of immune serum dilutions, inactivated 30 min at 56°C, multiple titrations were made with increasing amounts of unheated N-ch in each set. The amount that gave maximal titer without causing anticomplementary antigen control was optimum. Amount of N-ch was usually 5% in relation to antigen aliquot (Table I). (c) This was followed by 3 to 4 units antigen contained in 0.2 ml. N-ch was also added to antigen control. (d) Complement was added last. After 1 hour at 37°C,

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* Approved for publication as Scientific Journal Series Paper, Minn. Agric. Exp. Sta.

† This work was supported in part by grants from U. S. Dept. of Agric., U.S.P.H.S.

TABLE I. Titration for Optimal Amount of Normal Factor Required to Restore Titer of Inactivated Ornithosis Turkey Antiserum.

	Serum dilution				
N-ch, %*	1/32	1/64	1/128	1/256	Ag. control
0	0				Neg
2	4	4			"
3	4	4	3		"
5	4	4	4		"
7	4	4	4		"
10	4	4	4	2	2

* Calculated in relation to antigen aliquot.

the indicator system was added. Readings were made 30 minutes later or sooner if both serum and antigen controls were hemolyzed.

Results. Effect of temperature and kaolin treatment. The normal factor in chicken serum (N-ch) was destroyed at 56°C in 30 minutes but tolerated 45°C for 2 hours. It deteriorated at about the same rate as complement at room or refrigerator temperatures. It could be stored at -20°C for several weeks. The factor could be adsorbed out of diluted serum on kaolin and was found in water insoluble fraction of serum. The same substance was also present in immune serum and because of it, made titration of unheated serum possible. However, during serum dilution procedure, the factor was diluted out and fixation ceased even though antibody still might be present at higher dilutions (Table II).

Effect of serum dilution. Although addition of N-ch to antigen mixture has been used by us for some time, its lability to dilution was recognized only recently. For example, in procedure using 0.2 ml aliquots of serum dilution, antigen, and complement, 0.01 ml of N-ch, undiluted added separately gave a slightly higher titer than 0.1 ml 1/10 dilution and in turn both gave higher titers than when 0.2 ml of 1/20 dilution is added. The separate addition of 1/20 dilution gave titers comparable to antigen fortified with 5% N-ch. In each case the amount of N-ch was the same although activity was lost through dilution. The effect of dilution was also seen when serum was treated with kaolin. Very little of the factor was adsorbed from whole serum but at dilution of about 1/8 it was removed. The diluted adsorbed serum failed to fix complement as completely as heated serum. However, as was true with heated serum, the titer could be restored by replacing the labile factor (Table III).

TABLE III. Effect of Normal Serum on Heated or Kaolin Treated Antiserum and Ornithosis Antigen.

	Serum antibody titers			
	Unheated	Unheated + N-ch	Heated	Heated + N-ch
Serum				
Untreated	1/32	1/128	Neg	1/128
+ kaolin	1/16	"	"	"
½ + kaolin	1/16	"	"	"
¼ + "	1/ 8	"	"	"
⅛ + "	Neg	"	"	"

Replacement of labile factor in chicken antiserum was not as uniform as in turkey. There was evidence of zoning in certain sera even when unheated, which precludes use of this procedure for screening chicken sera until zoning can be blocked satisfactorily.

Discussion. The use of normal serum to induce or enhance immune reactions has been noted many times. Mathos and Kidd(5) observed lymphoma cells exposed to heated antilymphoma serum were not destroyed. However, if normal unheated serum was added, the cells failed to grow when transplanted. In

TABLE II. Effect of Heated or Kaolin Treated Normal Chicken Serum on Ornithosis Antibody Titrations.

		Serum dilutions					
Serum No.	Antigen treated	1/8	1/16	1/32	1/64	1/128	AgC
89*	Ag alone	4†					Neg
	+ N-ch untreated	4	4	4	4	4	"
	heated	4					"
	kaolin treated	4					"
23*	Ag alone	4	4				
	+ N-ch untreated	4	4	4	1		
	heated	4	4				
	kaolin treated	4	4				

* Turkey test serum, unheated. † 4 = complete inhibition; 1 = 25% hemolysis.

the review by Lamanna(6) on adhesion phenomenon, he pointed out the necessity for adding unheated normal serum to bring about adhesion reaction. Ross(7) discussing enhancement by normal serum of the cytotoxic action of antiserum on certain cancer cells, referred to normal serum factor as complement.

If this normal chicken serum factor is a component of complement, indications are that it is C′ 1 or associated with C′ 1. It is heat labile and found in water insoluble fraction of serum. Perhaps turkey antibody-antigen complexes cannot activate guinea pig C′ 1(8). However, it is difficult to understand why this labile factor must be added before the heated antibody and antigen combine. If chicken C′ 1, is substituting for guinea pig C′ 1, one would not anticipate the order of addition to be of such importance.

The normal factor also had some characteristics of properdin(9). It was adsorbed on kaolin, was destroyed at 56°C, tolerated 45°C for 2 hours and was found in water insoluble fraction of serum. Pillemer *et al.*(10) state that properdin was not destroyed at zero or –20°C. In contrast, this factor was inactivated after a few days at zero to 4°C and was greatly reduced in titer after several weeks at –20°C. Nor did this substance appear to form a complex with inulin(11). Neither whole serum nor serum diluted 1/10 lost activity after treatment with inulin in the range of 25 to 200 mg/ml of serum.

The N-ch factor appeared to be specific for systems in which turkey and chicken antiserum were used. It did not alter the titer of heated human, bovine, sheep, or in fact, pigeon antiserum nor did it always prevent zoning in all chicken sera.

Studies in progress will attempt to identify this normal component and to investigate a similar relationship in other serum species.

Summary. A factor in normal chicken serum when added to heated or unheated immune turkey serum-antigen complexes caused fixation of guinea pig complement. The factor was labile at 56°, unstable at room or refrigerator temperatures but remained active for several weeks at –20°C. It was found in the water insoluble fraction of serum and could be removed from diluted serum on kaolin.

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Received March 19, 1959. P.S.E.B.M., 1959, v102.

Antibody Response to Homografts. I. Technic of Lymphoagglutination and Detection of Lymphoagglutinins upon Spleen Injection.* (25220)

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There is considerable evidence that serum antibody responses of various types occur following transplantation of homologous tissues

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* This investigation was supported by research grant from NIH, PHS and Cancer Research Co-ordinating Comm., Univ. of Calif.

TABLE I. Lymphoagglutinating Activity of Sera from Chickens Injected with Pooled Homologous Splenic Cells.

Immunization				Lymphoagglutination titer (reciprocal) with lymphocytes from chicken No.: (4-fold dil. of serum)		
Total No. of spleens inj.	Time between inj. (days)	Time between last inj. and bleeding (days)	No. of positive agglutinations/No. tested	337	323	305
8	6 (Freunds adjv. added)	6	14/14			
30	43 (Freunds adjv. added)	16	6/8			
14	13-11-13-13 (Freunds adjv. added)	14	13/13	256	16	4
22	0	8, 12	11/11	64	256	64
22	0	8	3/3	16	4	”
22	0	8	2/3	64	0	16
20	8	19	9/10	”	64	64
8	11	14	9/9	”	”	”
8	11	14	13/13	16	4	4
62	8-9-8-8	8	9/9	1024	64	64
1	0	8-15	3/3*			
	Various		11/11			
	Total		103/107			
	Single skin graft	12-16	5/6*			
Controls:	Autografted with skin		0/9	0,0,0	0,0,0	0,0,0
			2/21	2	2	
	Untreated chickens		3/52			
Total controls			5/82			

* Lymphocytes for test from spleen or skin donor.

(1-3). However, there is little evidence that these humoral antibodies are causally connected with rejection of homografts(4-7). To reexamine this problem, we found that chickens injected with homologous splenic cells or given skin homografts regularly produce serum antibodies which will agglutinate the donor's lymphocytes. In demonstrating antibodies against homografted cells, the lymphoagglutinin test described here is thought to offer 2 advantages over hemagglutination and leucoagglutination tests used previously(6,8,9). First, there is substantial evidence that red cells lack some transplantation antigens (10). Second, the difficulty with non-specific agglutination found in leucoagglutination technics is avoided by using lymphocytes. It has been found that lymphocytes do not agglutinate non-specifically even after repeated centrifugation (350 g, 10 min) and resuspension.

Methods. Immunization. Pooled splenic cells from White Leghorn (WL) chickens were injected into New Hampshire (NH) chickens or vice versa. Both breeds of chickens were from a non-inbred commercial stock. The spleens after removal were decapsulated and cut with scissors, Hank's solution was then added, and cell aggregates were further broken down by aspirations with a syringe. Splenic cells were mixed with Freund's complete adjuvant in roughly equal proportions for subcutaneous injection into 5 animals. All other chickens were injected subcutaneously without the adjuvant. After repeated injections of splenic cells, the birds were bled for sera as indicated on Table I. For tests with single spleen, 3 WL chickens were splenectomized, the spleens were dissociated into separate cell suspensions, and each was injected subcutaneously into 3 different NH chickens. Immunizations with homologous skin were done by a single 3 x 5 cm orthotopic graft. Sera were stored at 4°C, and in a few cases frozen at –14°C. All sera were inactivated by heating at 56°C for 30 minutes. The technic of isolation of lymphocytes from blood of chickens has been reported(11). Briefly, it consists of first incubating heparinized blood for about 30 minutes at 37°C, when large numbers of

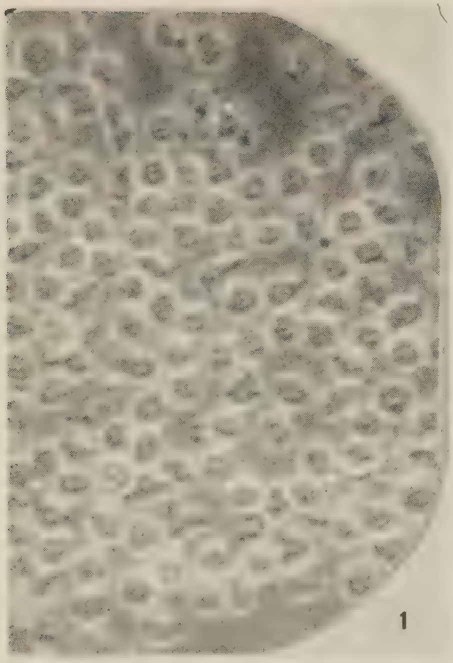

FIG. 1. Lymphocyte suspension. This is a suspension used for the lymphoagglutination test, prepared from blood of chickens by methods described in text. In this field, there are no red blood cells, no polymorphonuclear leucocytes, and 1 or 2 possible monocytes. All the other cells are lymphocytes (99%). (Phase contrast, 1000 ×.)

polymorphonuclear leucocytes and monocytes stick to wall of centrifuge tube. Blood is then centrifuged very lightly at speeds depending on height of blood in centrifuge tube so that red cells are packed to about ¾ of total volume (*e.g.* at 80 g for 10 min for 80 mm height of blood). The upper plasma layer containing lymphocytes is taken off carefully with pipette over thin, lightly packed, buffy coat. This step requires some experience in judging to what extent the pipette can be placed into the buffy coat without getting other white blood cells and red blood cells. The lymphocytes are concentrated by centrifuging at 350 g for 10 min and resuspended in Hank's solution. By these methods, suspensions of lymphocytes containing less than 1% contamination with other white blood cells have been routinely prepared. Red blood cell contamination has been less than 5% of lymphocytes. A photograph of an example of lymphocyte suspension is shown in Fig. 1. Total time required to prepare a suspension was 1-1½ hours. In all the tests, the lymphocytes were used within 3 hours after preparation. Although heparin seems to interfere with leucoagglutination technics(12,13), Bessis(14) found that heparin from certain sources does not cause non-specific agglutination. In our experience with heparin from Upjohn and Darwin laboratories, no non-specific clumps of lymphocytes were found at final concentrations of 10 u/cc to 50 u/cc. *Lymphoagglutination reaction* was carried out in: 1) chambers formed by microscope slide, a rubber gasket 1 mm thick, and coverglass; 2) agglutination slides with concavities 15 mm in dia. by 1.5 or 8 mm deep; or 3) test tubes—⅜″ dia. x 1¼″. To the test tubes and agglutination wells, 0.2 cc of serum dilution was added together with .02 cc of cell suspension (ca. 50,000/cu mm). All dilutions of sera were made with Hank's balanced salt solution. Upon incubation at 37°C for 2 hours, the cells were transferred onto a glass slide inside a wax ring .02-.04 mm in height and covered with coverglass. The wax ring served to prevent agglutinated clumps from being squeezed apart by pressure of coverslip. Agglutination was most easily detectable by using dark field illumination with 10 x objective (Fig. 2). All agglutination was then checked with phase contrast microscope employing a 43× objective, to determine whether clumps were lymphocyte clumps and not red blood cells or cells caught in microscopic pieces of clots or debris.

Results. Sera from chickens immunized by injection of splenic tissue almost invariably react with lymphocytes from other chickens (Table I). Thus 103 of 107 different serum-lymphocyte combinations tested showed positive lymphoagglutination. Substantial antibody titers were observed in most chickens injected with pooled spleens; in one instance a titer of 1:1024 was found. Spleens of one breed of chickens were used to immunize chickens of another breed. Sera of immunized chickens were tested with chickens of the spleen-donor breed and the recipient breed. Lymphoagglutination was obtained in 75 of 76 cases when antisera were tested with lymphocytes of spleen donor breed. However,

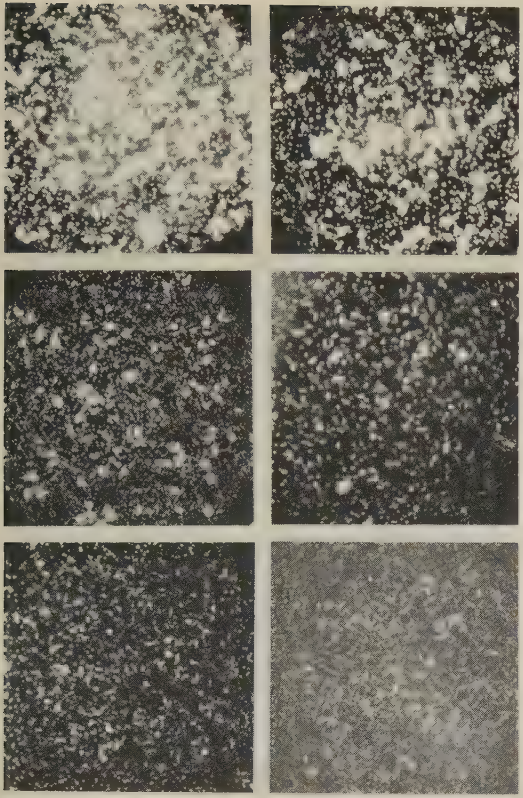

FIG. 2. Different degrees of lymphoagglutination (darkfield, 50 ×). Reading left to right: +4 agglutination, +3, +2, +1, ±, and —. In titration tests titer was taken as dilution of sera producing a +1 intensity of agglutination. About 98% of cells shown are lymphocytes. Occasional oval shaped cells about twice the size of lymphocytes are red blood cells.

even when antisera were tested with lymphocytes of recipient breed, lymphoagglutination was obtained in 28 of 31 cases. In other words, sera from NH chickens immunized with WL spleens reacted in most cases with lymphocytes from NH chickens. Although Freunds adjuvant was used in initial experiments to induce immunity, it was unnecessary for elicitation of lymphoagglutinins. In tests of 52 control sera from untreated birds, no reaction was obtained except in 3 cases. In these 3 instances it seemed that the clumping may have been caused by small clots formed around cells. It is also possible that natural agglutinins were responsible for the positive reaction. Sera from autografted chickens tested 2-3 weeks after grafting with lymphocytes from other chickens produced lymphoagglutination in 2 of 21 instances. In the 2 positive reactions, the titers of sera were 1 : 2.

Lymphoagglutinins were also demonstrated in chickens receiving a spleen or skin graft from one donor. Sera from 3 chickens injected with spleen, reacted in all 3 instances with the lymphocytes from the splenectomized donor. Sera of 5 of 6 chickens which were grafted with skin, also reacted with lymphocytes from the skin donor. Lymphoagglutinins produced after inoculation with splenic tissue appeared to be less specific than those detectable after rejection of skin grafts in that they reacted with 6 of 13 lymphocyte suspensions from chickens other than the spleen donor, while sera from skin-grafted animals reacted with lymphocytes from only 1 of 9 non-specific donors.

Discussion. In attempts to show that serum antibodies are at least in part responsible for rejection of homografts, probably the most direct evidence is that of passive transfer of immunity by serum antibodies. Many such attempts have been unsuccessful(15,16), although there are some recent reports of success(17,18). One of the difficulties in such tests is that large quantities of antibodies may be required to produce a gross visible effect on a graft. Using lymphocytes as target tissue, Woodruff and Forman were able to show that serum from rats either injected with lymph node cells or skin-grafted can cause a lowering of lymphocyte count in peripheral blood(19). Harris, Harris and Farber have recently shown that sera from rabbits immunized with pooled homologous leucocytes suppressed antibody forming capacity of passively transferred lymph node cells(20). It is likely, however, that dissociated cells would be more sensitive to small amounts of antibodies when tested *in vitro*. Hemagglutination tests(6,8) have the disadvantage that red cells are known to lack certain transplantation antigens since they do not evoke either immunity (10) or tolerance(21) to skin homografts. Recently, it has been clearly shown that hemagglutinating antibodies are not responsible for homograft breakdown by Mitchison and Dube(6) and Hildemann and Medawar(8). Leucoagglutinins appear in humans following blood transfusions(Dausset,22; Payne,23). Following rejection of skin homografts between different strains of mice, leucoagglutinins were detected against peritoneal exudate cells of the skin donor strain(Amos *et al.,* 9). In our preliminary trials to repeat such experiments with chickens, considerable difficulty was experienced in obtaining sufficient cells from peritoneal washings and in spontaneous clumping of cells. Since blood lymphocytes could be resuspended without aggregation even after repeated centrifugations, the lymphoagglutination test was devised. With this technic, as in the work of Amos, *op. cit.,* antibodies were detected in sera of animals which had been homografted with skin. Also, serum antibodies which caused lymphoagglutination appear in 96% of animals either injected with cells from homologous pooled spleens or a single homologous spleen. Sera from chickens of one breed injected with pooled spleens of another breed reacted with lymphocytes from chickens of both donor and recipient breeds. This rather wide specificity of antisera would be expected from our previous work showing that noninbred White Leghorn and New Hampshire chickens are antigenically almost as different among chickens of one breed as they are from chickens of the other breed(24). The sera from animals injected with splenic cells from a single donor also reacted with lymphocytes from chickens other than the spleen donor, thus indicating chickens of breeds tested share a considerable number of antigens. Lymphoagglutinins were present in sera of only 6% of normal chickens and 10% of autografted chickens. Since 4 of the 5 positive tests in these controls were obtained in our early experiments, they may possibly be due to technical error. However, neither natural agglutinins nor technical error can explain agglutination reactions found with 96% of sera from homografted animals. Our results indicate that titers reach a maximum about 3 weeks after grafting(25,26). The occurrence of these antibodies do not, of course, necessarily implicate them as causal agents of the homograft reaction. If it is true that leucocytes and skin cells share transplantation antigens in common(10), it would seem that antibodies reacting upon leucocytes may also react upon skin cells. To what extent these antibodies react on grafted cells *in vivo* and what role this may play in the rejection of homografts is yet to be determined.

Summary. An agglutination method for detecting antibodies against blood lymphocytes is described. By this method non-specific clumping of polymorphonuclear leucocytes and/or monocytes usually encountered in leucoagglutination tests is avoided. The humoral response following homografting in chickens was reinvestigated using this lymphoagglutination reaction. In 82 combinations of normal sera or sera from autografted chickens, and lymphocytes, lymphoagglutination was found in only 5 instances. On the other hand, 103 cases of positive agglutination were obtained in tests with 107 combinations of immune sera and normal lymphocytes. These immune sera, including one with titer of 1:1024, were from animals previously injected with pooled homologous spleens. Sera from animals injected with splenic cells or grafted with skin from one donor, also contained lymphoagglutinins against lymphocytes of spleen or skin donor chicken. These lymphoagglutinins also reacted in some instances with lymphocytes of chickens other than spleen or skin donor.

The authors wish to thank Drs. D. T. Imagawa and W. H. Hildemann for advice and valuable criticism of manuscript. We are also indebted to J. D. McClelland, C. C. Chamberlain, and M. A. Coulson for technical assistance.

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Received June 1, 1959. P.S.E.B.M., 1959, v102.

Comparative Threonine, Valine, Histidine, and Glucose Oxidation in Chicks.* (25221)

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There are very few reports of comparative studies on relative amounts of different essential amino acids which will be catabolized and oxidized, or synthesized into protein after ingestion by an animal. Mice injected intravenously with 0.7 to 2 mg of labeled amino acids(1) oxidized 28% of injected activity from glycine-1-C^{14}, 45% of that from L-histidine-2-imidazole-C^{14}, 55% of that from L-leucine-1-C^{14}, and 25% of that from L-lysine-1-C^{14} to expired CO_2 within 4 hours. Peak $C^{14}O_2$ production occurred within 30 minutes after injection. Fasted rats fed 25 mg of glycine-1-C^{14}(2) oxidized 50% of activity to CO_2 within 18 hours. Young turkey poults(3) had a maximum $C^{14}O_2$ production within 2 hours after subcutaneous administration of DL-lysine-2-C^{14} with total recovery of 20% of administered activity within 24 hours. Fasted phlorizinized rats given DL-lysine-6-C^{14} orally(4) oxidized 40% of activity to CO_2 in 8 hours. In a comparison of phenylalanine, tyrosine, and tryptophan metabolism(5), tracer doses of uniformly labeled amino acids administered orally to fasted rats showed a maximum $C^{14}O_2$ production during the first hour after administration. Within 8 hours, 45% of the C^{14} in tyrosine had appeared as CO_2, 20% of that from phenylalanine, and 25% of that from tryptophan. Tumorous mice injected intravenously with DL-tyrosine-3-C^{14}(6) oxidized 40% of the activity to CO_2 within 24 hours. Phenylalanine-deficient mice fed meals containing uniformly labeled L-phenylalanine(7) converted a smaller percentage of tracer doses to CO_2 than did normal mice when the meal contained a deficient level of phenylalanine, but a larger percentage when the meal contained a normal level of phenylalanine. This suggests that an animal tends to conserve an amino acid if it is deficient in its diet. This is also shown in the report that rats fed diets rich in methionine(8) oxidized 25% of methyl-C^{14} to CO_2 in 24 hours, but when diets contained a marginal level of methionine, only 6% of methyl-C^{14} appeared in CO_2 in the same time. Peak $C^{14}O_2$ production was reached 2 to 3 hours after oral administration of labeled amino acid in both cases. Since these reports involved various experimental animals, fed or fasted, with amino

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* Part of data have been taken from thesis submitted to Graduate Division of Univ. of California in partial fulfillment of requirements for degree of Doctor of Philosophy.

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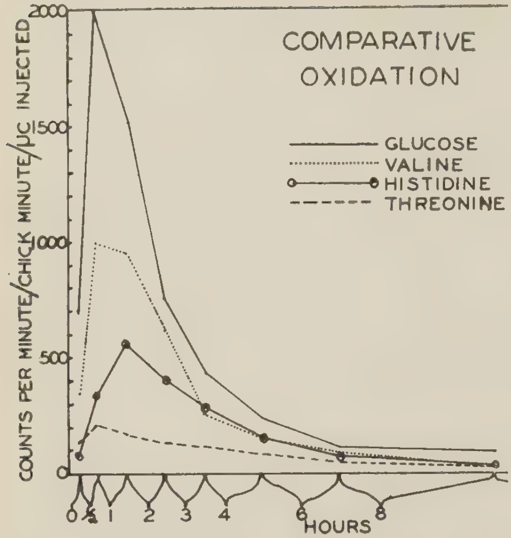

FIG. 1. Avg recovery of $C^{14}O_2$ from chicks after intraper. injections of uniformly labeled amino acids and glucose.

acids labeled in various positions, and administered by different methods, it is difficult to compare the data critically.

Methods. In this study of oxidation of essential amino acids, intraperitoneal injections of different amounts of solutions of L-threonine, L-valine, L-histidine, and glucose uniformly labeled with carbon-14 were given young chicks weighing 80 to 150 g. They had been previously fed either a complete purified diet based on crystalline amino acids, a diet lacking the amino acid injected, or a diet lacking all essential amino acids. The chicks were fed before being injected and feed and water were available in the metabolism chamber in which they were placed immediately after being injected. Respiratory CO_2 was trapped in NaOH, precipitated as $BaCO_3$ by addition of $BaCl_2$, collected on filter paper circles by filtration, dried, and activity determined by counting in a windowless, gas-flow counter (Tracerlab). The counts obtained were corrected for self-absorption, aliquot volumes used, and length of collection periods. They are expressed as counts/minute for each minute of collection time/microcurie administered. Validity of the intraperitoneal method of administration was checked by oral forced feeding of diet mixed with labeled threonine. Oral and intraperitoneal administration gave similar results.

Results. Comparison of average rates of $C^{14}O_2$ production by chicks after injection of labeled compounds is given in Fig. 1. The curves are averages for 4 or 5 chicks for each compound. Total recovery of administered activity during the 14 hour periods averaged 10% for threonine, 25% for histidine, 40% for valine, and 50% for glucose. In addition, peak $C^{14}O_2$ production varied among the 3 amino acids. Peak production from valine and threonine occurred close to one hour after injection, but peak production from histidine did not occur until nearly 2 hours after injection.

Different diets previously fed had little effect on oxidation of each amino acid or glucose. A diet lacking the labeled amino acid administered had little effect on the fraction of amino acid oxidized except possibly in the case of one lacking all essential amino acids. Even 10-fold differences in amounts injected from less than one mg to amounts above the daily requirements did not affect the fraction oxidized to CO_2. There was no apparent conservation of an amino acid lacking in the diet. Activity remaining in chicks at end of experimental period was much higher in blood, liver, and muscle picric acid-precipitable proteins of chicks given threonine than of those given histidine, whose activity was higher than that of those given valine. Almost all activity remaining in the chicks was in picric acid-precipitable proteins; only limited amounts were found in free amino acids or other compounds. Most activity in proteins was in the form of injected amino acids, only limited activity appearing in non-essential amino acids.

Discussion. The percentage of each administered amino acid which is rapidly oxidized was consistent even when the level of the amino acid in the diet was varied from very low to high levels. It was typical for individual amino acids. The low percentage of labeled threonine oxidized to $C^{14}O_2$ raises the possibility that oxidation of an amino acid is not a result of a "spillover" of an excessive level of the free amino acid pool into oxidative pathways. In these experiments, the amount of threonine administered was varied enough so that it should have affected the level of the free threonine pool but the percentage of administered threonine which was oxidized was not changed. The rapid appearance of radioactivity in the picric acid-precipitable proteins points to the possibility, at least in the case of threonine, of some metabolic route from administered amino acids to bound amino acids to the oxidative pathways possibly without participation in free amino acid pools.

Summary. When different amounts of uniformly labeled L-threonine, L-valine, L-histidine, or glucose were administered intraperitoneally to young chicks previously fed complete purified diets or diets lacking different essential amino acids, the total percentage of administered activity recovered as $C^{14}O_2$ during 14 hour experimental periods averaged 10% for threonine, 25% for histidine, 40% for valine, and 50% for glucose. There were large differences in the fraction of each amino acid incorporated into picric acid-precipitable protein. Peak $C^{14}O_2$ production occurred about one hour after administration of glucose, threonine, or valine; 2 hours after administration of histidine. Neither previous diet (complete or lacking the amino acid injected) nor 10-fold variations in amounts of amino acids administered appeared to affect fractions oxidized or synthesized into proteins.

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Received June 9, 1959. P.S.E.B.M., 1959, v102.

Comparative Passage of Calcium and Strontium from Plasma into Cerebrospinal Fluid in Man.* (25222)

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Comparative passage of strontium and calcium through various membranes is of importance in determining distribution of Sr^{90} in the body. *In vitro* ultrafiltration experiments with serum using Sr^{85} and Ca^{45} as tracers have shown that filtration of strontium is greater than that of calcium and that strontium is less completely bound to plasma proteins than calcium(1). Since passage of these ions through various membranes *in vivo* may differ from that *in vitro*, the relative transfer rates from plasma into different body fluids were studied. This report deals with passage of Sr^{85} and Ca^{45} from plasma into cerebrospinal fluid in man. The question whether cerebrospinal fluid is formed by ultrafiltration or secretion has been much argued(2). It was hoped that these tracer studies might also help clarify this point.

Materials and methods. Tracer doses of Sr^{85} (approximately 0.2 μc/kg body weight) or of Sr^{85} and Ca^{45} (approximately 0.2 μc/kg body weight of each) were administered as chlorides in single intravenous injections to 17 patients in whom lumbar puncture was to be performed for medical reasons. The intervals between injection of the tracers and lumbar puncture ranged from 5½ to 47 hours, at

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* Part of this work presented at 1957 meeting of Fed. Soc. Exp. Biol. It was supported by Atomic Energy Comm. grant.

† Post-doctoral Research Fellow, Nat. Found. for Inf. Paralysis, 1957-1958.

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TABLE I. Ratios of Sr^{85}, Calcium, and Phosphorus in Cerebrospinal Fluid Samples Obtained Approximately 24 Hours after Intravenous Administration of Sr^{85}.

Patient	Diagnosis	Protein CSF,* mg %	Protein Plasma, g %	Hr	CSF:* Serum ratios† Sr^{85}	CSF:* Serum ratios† Ca	CSF:* Serum ratios† P
1	Cancer of lung	25	5.8	23	.46	.52	.33
2	*Idem*	32	8.2	24	.33	.56	.37
3	Diabetes mellitus	32	6.1	”	.35	.56	.45
4	Cancer of cervix	75†	6.7	”	.54†	.54	.34
5	breast		6.8	”	.29	.51	.48
6	uterus		6.1	26	.18	.53	.48
7	”	78	5.8	”	.40	.52	.44
8	Hypernephroma	44	6.9	28	.40	.50	.38

* CSF = Cerebrospinal fluid.

† Previous paravertebral inj. of alcohol may have rendered spinal dura more permeable.

which time samples of cerebrospinal fluid and plasma were obtained. Concentrations of Sr^{85}, Ca^{45}, calcium, phosphorus, and total protein were determined in plasma and cerebrospinal fluid. Stable calcium could be determined only on those samples of cerebrospinal fluid not utilized for Ca^{45} analysis. Blood urea nitrogen was determined on the plasma of each patient and plasma proteins were determined by paper electrophoresis on most patients. Calcium was determined by the method of Kramer and Tisdall as modified by Clark and Collip(3), inorganic phosphorus by the method of Fiske and SubbaRow(4), total serum protein by the Kjeldahl method, a small correction being made for non-protein nitrogen, and cerebrospinal fluid protein by the biuret method. Sr^{85} was counted in a well scintillation counter and Ca^{45} in a gas flow counter following precipitation with carrier calcium as the oxalate, both methods having been previously described(5). Concentrations of Sr^{85} and Ca^{45} were calculated as percent of administered dose per liter and were expressed as ratios of cerebrospinal fluid to plasma.

Results. Table I shows cerebrospinal fluid to plasma ratios of Sr^{85} approximately 24 hours after intravenous administration of the tracer. The ratio of stable calcium in cerebrospinal fluid to that in plasma is close to the average value of 0.53. Values for Sr^{85}, on the other hand, are consistently lower, except for Patient 4, in whom the spinal dura might have been rendered more permeable by paravertebral injection of alcohol 3 hours prior to lumbar puncture.

Table II shows relative values of Sr^{85} and Ca^{45} in cerebrospinal fluid and plasma at different time intervals following simultaneous intravenous injection of the tracers. No appreciable difference in passage of the 2 radioisotopes was noted except in Patient 17, in whom a greater percentage of Ca^{45} than of Sr^{85} was found in cerebrospinal fluid after 47 hours.

Table III shows plasma levels of Sr^{85} and Ca^{45} following intravenous injections of the tracers. The data are in agreement with those obtained on a large number of patients studied with both tracers at this institution. Plasma levels of the 2 radioisotopes are practically identical during the 2 days following intravenous injection, so that comparison of cerebrospinal fluid/plasma ratios is meaningful despite considerable time lag in passage of the 2 radioisotopes into cerebrospinal fluid.

Calculations of the approximate rate of cerebrospinal fluid formation were made on the assumption that maximum concentration of calcium in cerebrospinal fluid was half that

TABLE II. Comparative Passage of Sr^{85} and Ca^{45} from Plasma into Cerebrospinal Fluid at Different Time Intervals following Intravenous Administration of the Tracers.

Patient	Diagnosis	Hr	CSF*: Serum ratios Sr^{85}	CSF*: Serum ratios Ca^{45}
9	Syphilis	5½	.10	.10
10	Multiple sclerosis	12	.22	.25
11	Cancer of lung	21	.42	.39
12	Polyarthritis	22	.37	.40
13	Peripheral neuropathy	22	.44	.43
14	Cancer of stomach	22	.34	.33
15	” ” esophagus	24	.42	.36
16	Diabetes mellitus	42	.45	.42
17	Cancer of lung	47	.34	.51

* CSF = Cerebrospinal fluid.

TABLE III. Plasma Levels of Sr^{85} and Ca^{45} at Varying Time Intervals following Intravenous Injection of the Tracers.

Patient	Time after inj. (hr)	% of dose/1000 ml plasma Sr^{85}	Ca^{45}
9	5½	2.77	2.55
10	½	4.13	4.30
	12	2.79	2.83
16	½	4.59	4.17
	15½	2.14	2.12
	42	1.37	1.49
17	½	6.09	5.67
	23	2.04	1.94
	40	1.38	1.43

of the plasma from which it was formed. This assumption is borne out by the data on stable calcium listed in Table I. It was also assumed that average Ca^{45} plasma concentration, from the time of injection of tracer, was twice the Ca^{45} concentration at n hours. Total volume of cerebrospinal fluid varies from 80-100 ml in different individuals(6). An assumed total volume of 100 ml was used in the calculations. Time of formation of the total amount of cerebrospinal fluid was calculated as equal to $\frac{n}{Ca^{45}\text{ in CSF}/Ca^{45}\text{ in plasma}}$, where Ca^{45} concentrations in cerebrospinal fluid and plasma were determined after n hours. Rate of formation of cerebrospinal fluid was then $\frac{100}{\text{time of formation}}$.

Using the 5½ hour value of Patient 9 in Table II, time of formation of cerebrospinal fluid was $\frac{5\frac{1}{2}}{0.10}$, or 55 hours, so that rate of formation was 100/55, approximately 2 ml/hour, or of the order of 0.03 to 0.04 ml/hour/kg body weight. Similar results were obtained by using the data of the other patients listed in Table II.

Discussion. Cerebrospinal fluid/plasma ratios of Sr^{85} and Ca^{45} were practically the same except in one patient in whom the ratio was greater for Ca^{45}. As Sr^{85} is less completely bound to plasma proteins than Ca^{45}, and ratio of Sr^{85} to Ca^{45} in ultrafiltration experiments *in vitro* is 3:2 or 5:4, it may be concluded that there is a preferential passage of Ca^{45} through the membranes involved in formation of cerebrospinal fluid. It therefore appears that cerebrospinal fluid in man is formed by secretion rather than by ultrafiltration.

The low content of inorganic phosphorus in cerebrospinal fluid compared to that in plasma has been previously reported(7), and in agreement with the data reported here, also indicates that cerebrospinal fluid may be formed by secretion. It has been argued that these low ratios are due to ultrafiltration followed by modification of the cerebrospinal fluid(2), possibly by reabsorption of some of the inorganic phosphorus. However, the data of Table II do not readily admit of this interpretation. Because of the low values of Sr^{85} and Ca^{45} in the cerebrospinal fluids of Patients 9 and 10, reabsorption of these ions should have had only a negligible influence. Nevertheless, Sr^{85} and Ca^{45} ratios were the same in both cases. If these ratios were due to ultrafiltration followed by considerable reabsorption, one would not expect an increase in the ratios of both radioisotopes with time (Table II).

No correlation was evident between rate of passage of Sr^{85} into cerebrospinal fluid and plasma or cerebrospinal fluid proteins. The factors which determine passage of the ions across membranes into the spinal canal varied considerably from patient to patient.

The rate of cerebrospinal fluid formation in dogs has been reported to be 0.2 ml/hr/kg(8), appreciably greater than the value here calculated for man. Rate of formation for dogs, however, was determined after complete drainage of cerebrospinal fluid, a highly unphysiological condition, so that the values obtained cannot be related to those in normal animals nor to human beings.

Studies carried out in man have indicated a rapid passage of deuterium into the subarachnoid space, the deuterium concentration in the spinal fluid equalling that of plasma in about 80 minutes(9). However, deuterium was probably involved mostly in exchange at the surfaces of brain, spinal cord, and cerebral ventricles, rather than in formation of new cerebrospinal fluid. Studies with Na^{24}, Rb^{86}, and P^{32}, as well as other radioisotopes, have revealed considerably lower equilibration rates than obtained with deuterium(8-10). The different rates of passage of various elements into cerebrospinal fluid point to the process of secretion rather than ultrafiltration.

Summary. Comparative rates of passage of strontium and calcium from plasma into cerebrospinal fluid have been studied in man following intravenous injection of Sr^{85} and Ca^{45}. Cerebrospinal fluid to plasma ratios of Sr^{85} and Ca^{45} were approximately the same except in one patient who showed a greater ratio for Ca^{45}. As a greater proportion of Sr^{85} than of Ca^{45} is unbound to protein, differential passage into the cerebrospinal fluid in favor of Ca^{45} appears to take place. The data indicate that cerebrospinal fluid may be formed by secretion rather than ultrafiltration. Rate of formation of fluid has been calculated to be approximately 0.03 to 0.04 ml/hr/kg of body weight.

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Received June 10, 1959. P.S.E.B.M., 1959, v102.

Effects of Antibiotics on Multiplication of L Cells in Suspension Culture.
(25223)

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A number of agents known to have an antitumor effect in animals or man were recently shown by Eagle and Foley(1,2) to be highly toxic against a number of cultures of human and animal cells. They suggested that determination of cytotoxicity in cell cultures might prove to be of value as a primary screen for detection of new carcinolytic agents. Their technic depends on the cytotoxic agent affecting ability of the cells to adhere to glass and to multiply under these conditions. During the past few years the method of Owens *et al.* (3) for growing mammalian cells in suspension culture has been reproduced (with modifications) in several laboratories(4,5), and it seemed to us that cells growing in suspension would be more suitable for study of the effects of cytotoxic agents than cells growing on glass surfaces. We have examined the effects of many substances on the L cell line of mouse fibroblasts growing in suspension culture and wish to report here on the effects of certain antibiotics on this cell line.

Methods. Stock cultures of the L cell line were grown in suspension in the modification of Eagle's medium(6) summarized in Table I. Approximately 200 ml of sterile medium and 20 million cells (taken from suspension cultures) were added to a sterile 500 ml flat-bottomed centrifuge bottle containing a sterile teflon-covered 2-inch magnet and capped with a black rubber stopper. The bottle was then placed on a magnetic induction stirrer located in a 37° incubator and the power adjusted so that the magnetized bar turned at 225 rpm. At the end of a 3-or 4 day incubation period

TABLE I. Composition of Medium Used for Growth of L Cells in Suspension Culture.*

Glucose	2.5 g	L-arginine • HCl	.021 g
NaCl	7.0	cystine	.012
KCl	.4	histidine • HCl	.008
$MgSO_4 \cdot 7H_2O$	.2	isoleucine	.026
Na_2HPO_4	1.44	leucine	”
D-biotin	.001	lysine • HCl	”
Ca pantothenate	”	methionine	.008
Choline chloride	”	phenylalanine	.016
Folic acid	”	threonine	.024
Nicotinamide	”	tryptophane	.004
Pyridoxal • HCl	”	tyrosine	.018
Thiamine • HCl	”	valine	.024
Riboflavin	.0001	glutamine	.3
Phenol red	.01	Potassium benzylpenicillin	.06
Methocel (4000 cp)	.3		
Calf serum	100 ml	Dihydrostreptomycin sulfate	.1
Pyrogen-free water q.s.	1 l.	Nystatin	50,000 units

* This medium is a modification of that described by Eagle(6).

the population (as determined by direct count using a hemocytometer) was usually between 120 million and 180 million cells. Aliquots were removed for inoculation of secondary bottles and for use in cytotoxicity tests. The cytotoxicity tests here described were carried out by growing the L cells in 1 x 6 inch test tubes capped with teflon-lined screw caps. Aliquots of sterile medium (Table I) were added to sterile flat-bottomed centrifuge bottles together with sufficient inoculum to give a population of about 100,000 cells per ml. The suspension was stirred with a magnetic stirrer for 1 hour to disperse the cells, then 25 ml aliquots were added to the sterile 1 x 6 inch screw-capped test tubes. Appropriate dilutions of sterile solutions of the antibiotics under study were added to the inoculated tubes, and the tubes were placed on a roller tube apparatus (New Brunswick Scientific Co., model TC-3) located in a 37° incubator. After 1 to 3 hours 2 ml aliquots were removed from each tube and cell population determined by direct count in the hemocytometer. These counts were taken as the "zero" time count, and after 3 days incubation a second series of counts was made. The multiplication factor shown on the ordinate of Fig. 1 was calculated from the increase in cell population over this interval. The multiplication factors in unsupplemented control tubes have varied from 6 to 9 over a series of 45 experiments. The amount of antibiotic needed to give 50% inhibition of growth was determined graphically from dose-response curves similar to those shown in Fig. 1. More consistent results were apparently obtained by direct count of the cell population than when increase in cell numbers was determined by a chemical method based on increase in protein nitrogen(7).

Results. Addition of solutions of various antibiotics to L cells growing in the modified Eagle's medium resulted in inhibition of growth with 10 of the 38 antibiotics studied. Those inhibiting growth and the concentrations of each needed to reduce growth to approximately 50% of that obtained in the unsupplemented control culture are listed in Table II. Those antibiotics which did not affect growth when added to L cultures at levels of 1 μg per ml are listed in Table III. Typical dose-response curves are shown in Fig. 1. Repeated experiments showed that the concentrations required for 50% inhibition of growth varied as much as 30% from one ex-

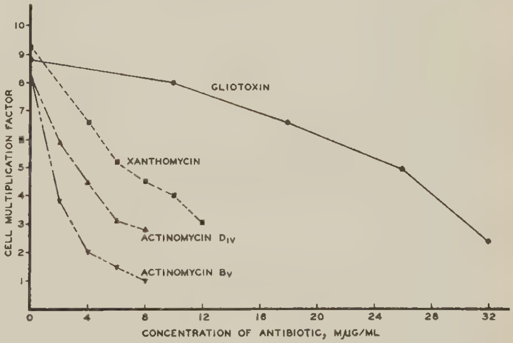

FIG. 1. Inhibition of multiplication of L cells by antibiotics.

TABLE II. Antibiotics Inhibiting Multiplication of L Cells in Suspension Culture.

Antibiotic	Approx. conc. giving 50% inhibition of multiplication of cells, mμg/ml
Actinomycin B_V	1.3
” D_{IV}	3
” $B_{(mixture)}$	4.6
Xanthomycin	7
Cycloheximide	33
Gliotoxin	36
Mitomycin C	41
Thiostrepton	70
Patulin	154
Hygromycin	1300

TABLE III. Antibiotics Not Affecting Multiplication of L Cells in Suspension Culture.*

Amphotericin A
" B
Aspergillic acid
Bacitracin
Benzylpenicillin potassium
Carbomycin
Chartreusin
Chloramphenicol
7-Chlortetracycline • HCl
Citrinin
Colistin
Cycloserine
Dihydrostreptomycin sulfate
Erythromycin
Fumagillin
Gramicidin
Griseofulvin
Methymycin
Neomycin B sulfate
Nystatin
5-Oxytetracycline • HCl
Polymyxin B sulfate
Subtilin
Tetracycline • HCl
Tyrothricin
Vancomycin
Vernamycin
Viomycin sulfate

* Non-inhibitory when added to medium to give a concentration of 1 μg/ml.

periment to the next; this variation was reduced when several replicate tubes were started at each antibiotic concentration. Of the substances tested that inhibited growth the actinomycins and xanthomycin were significantly more cytotoxic than any of the others. Experiments with concentrates obtained from fermented media containing cytotoxic substances showed that the dose-response curves could be used in estimating the potency of the cytotoxic factors present.

Examination of the literature shows that the cytotoxicity of only a few antibiotics have been examined in tissue culture. Eagle and Foley(1) found that approximately 60 $m\mu g$ per ml of actinomycin D were required to inhibit growth of 6 different cell lines of human and mouse origin. Whether the differences in activity of actinomycin D and the actinomycins listed in Table II is due to the chemistry or the sensitivity of the cell lines is unknown. Smith(8) has reported that approximately 100 $m\mu g$ per ml of cycloheximide is required to inhibit growth of the KB cell line. This is somewhat more than the 33 $m\mu g$ per ml that we need to inhibit the L cell line.

Summary. In a study of effects of antibiotics on multiplication of L cells in suspension culture, positive inhibition of multiplication was found when less than 1 μg/ml of any one of the following were added to the media: actinomycins B, B_V, or D_{IV}; cycloheximide; gliotoxin; hygromycin; mitomycin C; patulin; thiostrepton; and xanthomycin. Actinomycin B_V was the most active of the group tested as 1.5 $m\mu g$/ml was sufficient to cause a 50% reduction in growth of culture. While the shape of dose-response curves varied with different inhibitors, it was possible to use the linear portion as the basis of a method for quantitative estimation of concentration of inhibitor present. This method may be useful in determining the presence of cytotoxic metabolites formed in microbial fermentations.

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Received June 24, 1959. P.S.E.B.M., 1959, v102.

Influence of Fighting on Leukemia in Mice.* (25224)

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It has been observed, in leukemic strains of mice, that lymphoid leukemia occurs less frequently and later in males(1,2). This observation has been confirmed in our subline of inbred AK mice, in which: 1) incidence of leukemia is 15% less in males; 2) average age at death from leukemia is 315 days in males and 281 in females, a highly significant difference. Such a difference of susceptibility is commonly attributed to sex hormones. Estro-

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* This work was supported by grant of Nat. Cancer Inst. of Canada.

TABLE I. Influence of Fighting on Leukemia in AK Mice.

Groups	No. of mice	Age at death (avg days)	Death from leukemia: No. of mice	Death from leukemia: Age at death (avg days)	Not leukemic (%)
Females in colony	437	284.3 ± 4.5*	388	281.3 ± 4.1*	5.5-11.2
Males " "	249	329.3 ± 7.9	182	315.4 ± 6.2	17.7-26.9
" non-fighting (isolated)	40	295.3 ± 13.8	33	286.8 ± 11.3	2.5-17.5
" fighting ''beaten''	88	350.4 ± 13.4	66	331.8 ± 13.2	9.1-23.6
" " ''beaters''	30	375.2 ± 21.3	21	329.6 ± 13.9	16.7-30.0

* Stand. error of mean.

gens promote leukemia, whereas testosterone inhibits the disease; conversely, castration may stimulate leukemia in males and decrease it in females(1,2). Since female hormones or lack of androgens increase leukemia, and male hormones or lack of estrogens diminish it, females are believed more susceptible because they possess estrogens. This interpretation, though logical, was not entirely satisfying. It appeared that some other explanation could be added, or substituted. In several strains of mice, including the AK, males fight, sometimes savagely, even killing one another. It was assumed that such fighting could influence leukemia. Hence, the following experiment was set up.

Procedures. Forty male AK mice were isolated, each in a separate cage, before they began to fight, that is, when less than 65 days old. In a second group, males were placed 4-6 to a cage, to give them a chance to fight. When scuffling was noticed, this was watched closely and when a mouse showed traces of battle, was bitten and wounded, it was isolated. We took a larger number of animals in this group than in the first, for fear that some of them would be killed in fight and lost for the experiment; we ended with 88. It was more difficult to obtain a third group, *i.e.*, males that were not beaten, but beat and conquered the others. These are less numerous than their weaker companions, there is usually one per cage; they are hard to recognize, as they bear no trace of struggle: one has to watch for hours to be sure. Thirty were gathered. All mice were kept under observation until death.

Results. A difference was found between the animals that were isolated early and did not fight and those that fought. Unfighting males died of leukemia at 286.8 days, practically at the same age as females (Table I); moreover, the proportion of non-leukemic animals was low and comparable to that of females. There are 2 figures in the last column (Table I), because some individuals showed slight inconclusive signs of leukemia, or some had both leukemia and other diseases: although these mice died *with* leukemia, it could not be decided if they died *of* it. If they are reckoned as leukemic, we consider the first figure; if not, we consider the second. In the columns showing death from leukemia, they were regarded as not leukemic. Males that fought and were defeated died of leukemia at 331.8 days, a significant difference of 45 days over the non-fighting; the percentage of animals without leukemia was also higher in this group. As for the fighters that beat the others, their survival time was longer and the proportion of non-leukemics higher than in non-fighting mice. Their survival was also longer than that of beaten fighters, but the difference is not significant and, if only death from leukemia is considered, there is no difference; however, the percentage of non-leukemics is higher.

In the Table, mice termed "in colony" consist of animals that lived past the age of one month and, for the males, either did not fight or fought not enough to be separated as fighters. It is understood that females do not fight.

Comments. In brief, male AK mice that did not fight died of spontaneous leukemia at essentially the same age and in the same proportion as females, whereas males that fought had a lower and later incidence of leukemia. Therefore, if male mice in general become leukemic less often and later than females, it is not only because of sex hormones. Other causes contribute to this effect and fighting may be one of them. The influence of fighting could be explained through the action of adrenal hormones. Damage suffered by beaten mice, wounds, sequels of the wounds, such as sterilization and urinary calculi (some mice are sterilized by bites and other lesions of scrotum and penis; some develop stones in the bladder, apparently as a result of interference with micturition), these and other injuries initiate ACTH discharge. This elicits a secretion of corticosterone or similar hormone, which, as is known, retards and decreases leukemia. This situation is comparable to what happens in other hard conditions, such as pregnancy or underfeeding. Pregnancy retards leukemia in AK mice and the same explanation has been proposed(3). In underfed mice, leukemia is also reduced(4,5).

Incidence of leukemia and survival time of fighters that conquered the others were expected to fall between those of non-fighters and of beaten mice, because they underwent the strain of battle, but suffered almost no wounds nor the frustration of being vanquished. Actually, their survival was about the same as that of the beaten. Incidence of leukemia was appreciably less; perhaps they are stronger than the others in many respects, not only in combat behavior, but also in resistance against disease; which is further intimated by the fact that their survival time in general, not only as regards leukemia, is the longest.

The figures representing age at death from all causes (Table I) may give the impression that the influence of fighting is exerted on life span in general and not concerned with leukemia in particular. Indeed, there is no definite answer to the question whether fighting acts specifically on leukemia or on any cause of death. However, it seems to have a special effect on leukemia, because differences in survival are significant when death from leukemia is considered, but not significant in nonleukemic animals.

It is possible that androgens have an influence. All male groups were composed of intact animals, with testes and therefore testosterone; hence, the differences do not appear to come from sex hormones. But fighting itself could increase androgen production. Testosterone stimulates the fighting instinct. Fighting in turn could stimulate hormone secretion, or action. Thus, by reciprocal causation, testosterone would be notably increased and could inhibit leukemia. We have no evidence for this at present.

Another idea would be that early isolated mice are not disfavored by escaping the hardships of battle, but by isolation itself. In some species, resistance against different noxious agents, including cancer(6), is higher in animals living together than in isolated individuals. It is difficult to investigate this in male mice. If put with males, they fight; with females, it is hard to decide whether any observed effect is due to escaping fight, to sexual activity, or to the company of the female. Experiments are under way with castrated mice to ascertain this point and to establish the influence of testosterone.

Whatever the interpretation, spontaneous leukemia was delayed and diminished in males that fought. This fact must be remembered when differences are noted between male and female mice with respect to leukemia. In designing experiments with males, one should use only animals isolated early after weaning; otherwise, fighting may alter the results.

Summary. 1) In leukemic strains of mice, lymphoid leukemia is known to occur less often and later in males than females. However, in our AK mice, males that were isolated early in life and did not fight died of spontaneous leukemia at essentially the same age and in the same proportion as females, whereas fighting males died significantly later and in a lower percentage. Hence the difference between sexes is not explained by sex hormones only. An interpretation is proposed, based on adrenal corticosteroid secretion. 2) Fighting activity should be taken into account as a factor in pathogenesis of mouse leukemia and in the difference of incidence and survival time between male and female mice.

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Received June 22, 1959. P.S.E.B.M., 1959, v102.

Prevention of Peritoneal Free (Ascites) Tumor Cell Growth and Implantation by Pretreatment with Radioactive Yttrium.* (25225)

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It is well known(1,2) that thoracic and abdominal malignant tumors extending into pleural or peritoneal cavity may induce there accumulation of an exudate containing numerous malignant, frequently mitotic cells released from the tissue. Some of these cells eventually implant into the cavity wall and give rise to secondary growth of "coelomic metastases"(3). Müller reported(4) "undoubtful therapeutic results" of intraperitoneally injected colloidal Au^{198} in 8 female patients with "carcinomatous peritonitis." We showed(5) that peritoneal carcinosis (later designated as "ascites tumor") of the mouse(6) regressed completely after a single injection of colloidal Au^{198}; we obtained analogous results by similar treatment of free tumor cell growth in the pleural exudate(7). Later experiments(8,9,10) suggested the use of radioisotopes for inactivation of tumor cells remaining in healthy tissues or spilled there after tumor removal. We recently investigated the possibility of preventing growth and implantation of cells separated spontaneously or by trauma from abdominal tumors and entering peritoneal cavity. Accordingly, we reversed the procedure of our earlier experiments by injecting a radioisotope intraperitoneally into mice before and not after tumor inoculation by the same route. Eventual effect on tumor cells was checked by comparison of the amount of effusion, its content of free tumor cells and the extent of implantation in experimental and control animals. The results are recorded below.

Material and methods. a) *Mice and tumor strain.* Swiss Albino mice (Albino Farms, Red Bank, N. J.) females of 24 to 27 g weight were used. Krebs-2 carcinoma was maintained as "ascites tumor" by several intraperitoneal transfers. b) *Tumor cell count in ascitic fluid.* Methods of isotope injection, of inoculation with requisite numbers of tumor cells, of exudate withdrawal from the cavity and of staining were described previously (5,7). Standard pattern of implantation in female mice (periuterine localization) was obtained(6). c) *Isotopes.* In preliminary experiments we have tested on parallel lines preparations of short-living and pure beta emitting isotopes: $CrP^{32}O_4$, $Y^{90}PO_4$, and $ZrP^{32}O_4$ obtained from Abbott Laboratories, Oak Ridge, Tenn. Since most consistent results were produced with $Y^{90}PO_4$, this isotope was selected for experiments presented below. Decayed (no radioactivity detected by standard counters) isotope was given to control mice for assaying any chemical effect of the compound independent from its radioactivity. d) *Amount of ascitic fluid.* Amount of exudate was estimated as proportionate to volume of fluid obtained by insertion of a glass capillary in peritoneal wall: $+++ = 0.5$ ml or more; $++ = <0.5$ but >0.1 ml; $+ = <0.1$ ml; $0 =$ no fluid. The reliability of this estimate was shown by comparison with amount of exudate measured at autopsy. e) *Recording spontaneous implantation.* Extent of implant growth of periuterine localization (6) was graded as follows: $0 =$ no macroscopic implants; $+ =$ small nodules (less than 2 mm in diameter); $++ =$ tumor (2-5 mm in longer diameter); $+++ =$ growth

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* This investigation was supported by grants from U. S. Atomic Energy Comm. and Damon Runyon Fn.

TABLE I. Effect of Intraperitoneal Pretreatment with $Y^{90}PO_4$ on Accumulation of Ascitic Fluid and on Growth and Implantation of Its Free Tumor Cells.

Schedule of pretreatment			Findings in peritoneal cavity		
Series No.	Dose of inj. isotope (mc)	Days between isotope inj. and tumor inoc.	Amount of ascitic fluid	Conc. of tumor cells (thous./ml) in ascitic fluid	Extent of implantation from fluid into peritoneum
1	.25	1	++ ++ - +++	38.9 14.3-40.1	+ + - +++
2	.25	3	+ 0 - ++	16.9 4.7-22.2	+ + - ++
3	.25	6	+++ ++ - +++	43.7 34.8-59.1	++ ++ - +++
4	.25	Controls	+++ ++ - +++	61.4 51.4-71.2	+++ ++ - +++
5	.15	1	++ ++ - +++	40.8 33.1-59.8	++ ++ - +++
6	.15	3	++ + - +++	29.3 12.5-47.4	++ + - +++
7	.15	6	++ ++ - +++	61.6 39.5-71.0	++ ++ - +++
8	.15	Controls	+++ ++ - +++	59.9 51.4-67.9	+++ ++ - +++

Avg and variation extremes are given for each group (20 mice). Technics of cell counts, of fluid volume estimates and of grading the extent of implantation were recorded in *Material and Methods*. Each of two groups of controls included 20 untreated mice and 5 treated with completely decayed isotope (no activity detectable by routine counters).

larger than ++. f) *Pattern of experiments.* Graded doses (0.1, 0.2, 0.25, 0.3, 0.4 millicuries) of $Y^{90}PO_4$ were injected intraperitoneally into several series of mice. Three or 6 days later, these mice were inoculated with standard doses (10^5) of Krebs-2 tumor cells; 3 to 4 days later their ascitic fluid was withdrawn, its volume was estimated and the number of tumor cells per ml were determined; a day or 2 later, the animals were sacrificed and inspected for presence of implants in the peritoneum; sections of their peritoneal wall were examined for pathological changes and for presence of microscopic implant growth.† The results are recorded below.

Results. Lethal toxicity of the isotope. Preliminary experiments have shown that intraperitoneal injection of 2 ml of 0.85% NaCl solution immediately before injection by the same route of 0.3 mc of isotope in a volume of 0.2 ml was followed by death of less than 5% of mice within 10 days; the same dose of isotope alone was lethal for about 50% of animals. Dose of 0.25 mc injected into the cavity previously injected with 2 ml of saline was rarely lethal, but without saline it was lethal for 15% of mice. Intraperitoneal inoculation of tumor cells increased mortality in both groups pretreated with 0.3 mc but not in the group given saline solution and 0.25 mc. Accordingly, we have recorded in Table I only the results obtained with saline and the latter dose and, for comparison, with much lower isotope dose of 0.1 to 0.15 mc.

It appears that the optimal intraperitoneal dose of $Y^{90}PO_4$ was very close to 0.25 mc (series 1 and 2 of Table I) injected into saline filling the cavity. Lower doses (series 5 and 6) affected only slightly the results of inoculation; higher doses (0.3 mc) were not more efficient, but less safe.

The optimum interval between pretreatment with isotope and tumor inoculation was 3 days (Table I) in series 2 and 6, *i.e.*, maximum effect on tumor growth was obtained in these series as compared with series 1, 3, 5 and 7. Growth initiated after 6 days (series 3 and 7) was influenced by pretreatment, as compared with controls (series 4 and 8) only in few mice (higher minimum levels than in controls).

The most conspicuous effects of the optimal

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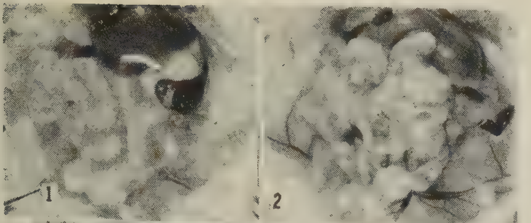
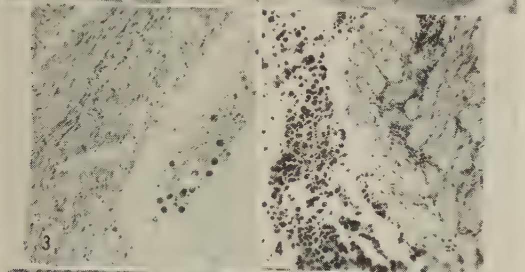
† Sid Cox, Medical Photographer, Vanderbilt Univ. Med. School, prepared the photographs.





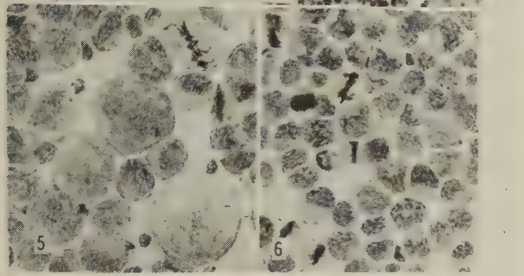


FIG. 1 and 2. Peritoneal implants 6 days after inoculation of 10^5 Krebs-2 tumor cells. Fig. 1, pretreated with 0.25 $Y^{90}PO_4$; Fig. 2, control.

FIG. 3 and 4. Peritoneal wall 4 days after inoculation of 10^5 Krebs-2 tumor cells. H + E; × 64. Fig. 3, pretreated with 0.25 $Y^{90}PO_4$; Fig. 4, controls.

FIG. 5 and 6. Free tumor cells in ascitic fluid 4 days after inoc. of 10^5 Krebs-2 tumor cells. Aceto-Orcein; × 128. Fig. 5, pretreated with 0.25 $Y^{90}PO_4$; Fig. 6, controls.

pretreatment method (Table I, series 2) were the decrease in the amount of ascitic fluid and reduction of macroscopically (Fig. 1 and 2) and microscopically (Fig. 3 and 4) visible implantation. Free tumor cells in the fluid appeared (Fig. 5 and 6) swollen with highly distorted mitotic figures. Intraperitoneal transfer of a standard amount of this fluid (0.2 ml) into new mice (biological test of viability) induced sizable growth considerably later (not before the 15th day) than did the fluid from controls (not later than on the 4th day).

Sections from peritoneal wall (serosa, subserosa and muscle) of pretreated and inoculated mice showed edema of connective tissue (Fig. 3). Variable numbers of tumor cells were scattered or arranged in clumps on the serosa. No edema was noted in controls in the same stage of tumor growth (Fig. 4); tumor cells in their serosa not only were more numerous but they spread into subserosa and infiltrated interfibrillar spaces of the muscle.

In a group of mice, pretreatment with 0.25 mc was supplemented 2 days after inoculation with a low dose (0.1 mc) of the same isotope. The results (Table II) showed that additional injection did not improve the effect of pretreatment on fluid accumulation and implant growth, but it did achieve extensive or complete destruction of free tumor cells in a number of the animals. The implication of these findings is discussed below.

Discussion. Occurrence of maximum growth inhibition in tumors inoculated 3 days after pretreatment, *i.e.,* after substantial decay of the isotope *in vivo* (half life of $Y^{90} = 65$ hours) suggested an indirect mechanism of damage to peritoneal growth by radioactivity and some direct effect on tumor cells. Free tumor cells and, in particular, their mitotic forms were considerably altered (Fig. 5 and 6) but in no instance were all cells destroyed; on the other hand, accumulation of ascitic fluid and growth of implants were considerably reduced with only a few exceptions. The latter effect may be attributed to edema of irradiated tissue disrupting fluid circulation, but it is also conceivable that irradiated peritoneal elements offer resistance to tumor cell invasion. Andrews, *et al.*(11,12) suggested that "some unknown mechanism in action, possibly upon surfaces not involved with tumor may be important" for therapeutic action of Au^{198} in human peritoneal carcinosis. Whatever is the nature of tissue reaction induced by radioactivity and opposing invasion of peritoneum by tumor cells and accumulation of ascitic fluid, it develops gradually, reaches its maximum effect on the 4th day and appears to be almost without effect about the 7th day.

In animals pretreated with 0.25 mc of Y^{90} PO_4, a low dose (0.1 mc) of the same isotope given 1 or 2 days after inoculation destroyed free tumor cells in higher numbers than did post-treatment alone (Table II, Series 5). It may be presumed that tumor cells altered or damaged by pretreatment were an easily vul-

TABLE II. Effect of Low Dose of $Y^{90}PO_4$ on Krebs-2 Free Tumor Cells Growing in Ascitic Fluid of Mouse Pretreated with Higher Dose.

Schedule of treatment			Findings in peritoneal cavity		
Dose (mc) of pretreating inj.	Dose (mc) of treating (after inoc.) inj.	Days between inoc. and treating inj.	Amount of ascitic fluid	Conc. of tumor cells (thous./ml) in ascitic fluid	Extent of implantation from fluid into peritoneum
.25	.1	2	+ 0 - +	5.8 1.5-9.4	+ + - ++
.1	.1	1	++ + - ++	19.8 11.6-43.5	++ + - +++
.1			++ ++ - +++	49.2 42.1-55.8	++ ++ - +++
	.1		++ ++ - +++	37.1 28.4-44.6	++ ++ - +++
Untreated controls			+++ ++ - +++	52.6 41.1-58.1	+++ ++ - +++

Twenty mice in each group. For each group, avg and variation extremes were recorded. Doses of 10^5 Krebs-2 tumor cells were inoculated to all animals. For technic of cell counts, of fluid estimate and of implants grading, see *Material and Methods.* Fluid was withdrawn for tests 24 hr after last inj. of isotopes: autopsies were performed 2 days later.

nerable target for even a small additional irradiation dose.

The association of pretreatment and post-treatment combined advantages of both methods, *i.e.,* inhibition of fluid accumulation and cell implantation was combined with extensive destruction of free tumor cells. Perhaps the principle of combined pretreatment and post-treatment with an isotope may be developed, with suitable modifications, into a method for prevention of transcoelomic metastases in patients with operated abdominal tumors. At present we are testing a combination of pretreatment with Y^{90} and of post-treatment with chemotherapeutic agents.

Summary and conclusions. (1) Doses of 0.25 mc of radioactive yttrium phosphate ($Y^{90}PO_4$) were injected into peritoneal cavity of Albino mice immediately following administration of 2 ml of 0.85% NaCl solution; on 3rd to 4th day 10^5 cells of Krebs-2 Ascites Tumor were inoculated by same route into pretreated animals and normal controls; their accumulated fluid was withdrawn after 3 or 4 days for estimate of its volume, tumor cell counts and smears; they were sacrificed for autopsies 1 or 2 days later. (2) Amount of ascitic fluid and extent of implant growth were considerably reduced in pretreated animals as compared with controls; free tumor cells in the fluid appeared enlarged, altered (distorted mitoses) and less viable, after transfer into new mice, than in controls; decrease of their concentration in the fluid was not as rapid and extensive as in animals treated with an isotope after inoculation in previous experiments. (3) Cell destruction was increased by injection of 0.1 mc into pretreated mice. (4) It was concluded that pretreatment with radioactive yttrium increased resistance of the peritoneum to invasion with tumor cells, thus decreasing implantation of these cells and exudation of the fluid. (5) It was presumed that the principle of this method may be eventually applied for prevention of "transcoelomic metastases" from some abdominal tumors, before or after their removal.

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Received June 25, 1959. P.S.E.B.M., 1959, v102.

Prevention of Salt Hypertension by Propylthiouracil Treatment in Rats.* (25226)

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The anti-thyroid drug, propylthiouracil, has been used successfully in rats to prevent development of renal hypertension(1,2); desoxycorticosterone-acetate induced hypertension(3) and adrenal-regeneration hypertension(4). Our studies were to determine whether this drug would also prevent a fourth type of experimental hypertension, namely that accompanying administration of hypertonic NaCl solution(5,6).

Methods. Holtzman rats were kept 2 or 3/cage in thermoregulated room at 25 ± 1°C, illuminated from 8 a.m. to 6 p.m. Blood pressures and body weights were measured during one-week control period and weekly thereafter for duration of experiment. Systolic blood pressure was measured by the microphonic manometer technic(7) but without anesthesia, as described previously(8). Immediately after the control period, 15 male rats (Exp. 1) were given 1.75% NaCl solution and 12 female rats (Exp. 2) 1.9% NaCl solution as sole drinking fluid. Eight rats in Exp. 1 and 6 rats in Exp. 2 received in addition ground Purina chow† containing 0.1% propylthiouracil (PTU)‡ The remainder of the rats received ground chow without the drug. After 11 weeks, all rats in Exp. 1 were killed by ether and heart, kidneys, testes, seminal vesicles, prostate, thyroid, thymus and adrenal glands were removed, trimmed, drained of excess fluid or blood, blotted on filter paper and weighed on torsion balance. Results are expressed as ratio of organ weight to body weight (mg/100 g B.W.). Since all rats in Exp. 2 lost nearly 50 g in 3 weeks, 1.75% NaCl solution was substituted for 1.9% NaCl solution. The substitution seemed to ameliorate weight loss. Only 4 rats in each group survived the total 14 weeks, then they were killed by ether and adrenals, thyroid, thymus and ovaries were removed and weighed as described above. Simultaneous means of control and experimental groups were compared using the "t" test for the 95% confidence limit (9).

Results. Fig. 1A illustrates that 1.75% NaCl solution given male rats produced a marked elevation in systolic blood pressure from 143 mm Hg to 165 mm Hg at end of Exp. 1. In contrast, rats receiving PTU in addition showed a decrease from 138 mm Hg to 128 mm Hg. Differences were significant ($P = .02$) by the second week and highly significant ($P < .01$) by 5th week. Mean body weight of rats receiving chow and salt fell during the experiment while that of PTU-treated group remained constant (Fig. 1B).

Systolic blood pressures of female rats in Exp. 2 showed results similar to, but more variable than, those of comparably treated groups of male rats in Exp. 1 (Fig. 2A). Control, salt-treated rats increased mean systolic blood pressure from 137 mm Hg to approxi-

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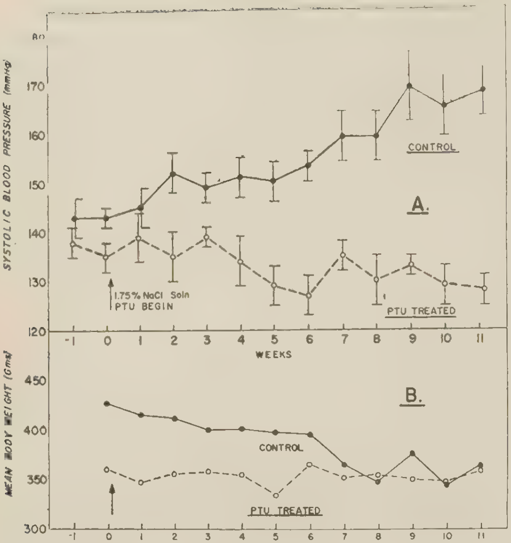
* Supported by Grants from Nat. Heart Inst., Bethesda, Md., and The American Heart Assn.

† Analysis of Purina laboratory chow by wet ashing with concentrated nitric acid and subsequently determining sodium and potassium content by flame photometry indicated a sodium content of 127 mEq./Kg and potassium content of 218 mEq./Kg.

‡ 6-n-propyl-2-thiouracil, Nutritional Biochemicals Corp., Cleveland O.



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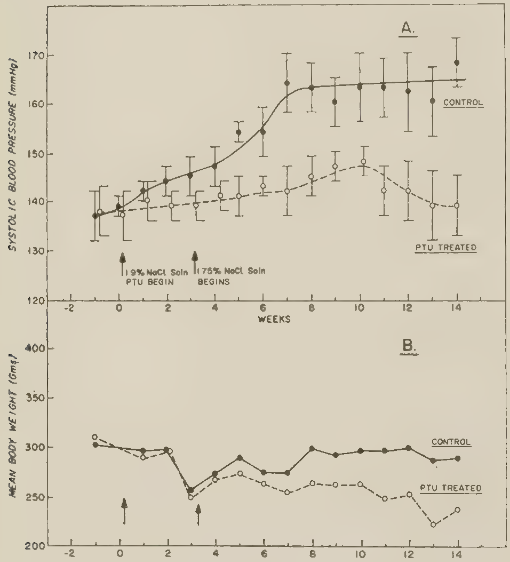

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FIG. 1. A. Mean systolic blood pressures of control (●) and propylthiouracil treated (○) male rats throughout experiment. Both groups of rats were given 1.75% NaCl solution as the sole drinking fluid. ± 1 stand. error is set off at each mean. B. Mean body wts of control (●) and propylthiouracil-treated (○) male rats throughout experiment.

FIG. 2. A. Mean systolic blood pressures of control (●) and propylthiouracil treated (○) female rats. Both groups of rats were given 1.9% NaCl solution for 3 wk and then switched to 1.75% NaCl solution for remainder of experiment. ± 1 stand. error is set off at each mean. B. Mean body wts of control (●) and propylthiouracil-treated (○) male rats throughout experiment.

mately 164 mm Hg in 14 weeks. PTU-treated rats showed a maximal rise to 148 mm Hg (10th week). Thereafter, blood pressure fell and by end of experiment was the same as during control period. Differences between the 2 groups were of limited significance ($P<.05$) beginning with week 5 (with exception of weeks 9 and 10).

After change-over from 1.9 to 1.75% NaCl solution, control rats in Exp. 2 regained their lost weight and maintained a relatively constant body weight (Fig. 2B). PTU-treated rats continued to lose weight during the experiment in contrast to their male counterparts in the first experiment.

In Exp. 1, ratios of heart and kidney weight to body weight of the PTU-treated group were significantly ($P<.01$) smaller than those of comparable controls while thyroid and testis weight ratios were significantly larger (Table I). Seminal vesicle, prostate, thymus and adrenal weight ratios of PTU-treated rats were similar to those of controls. Results in female rats of Exp. 2 were similar except that gonad to body weight ratio of the latter was reduced significantly ($P<.01$) by PTU-treatment (30 ± 6.1, control, and 16.1 ± 2.7, PTU).

Discussion. Hypertension developed in male and female rats fed commercial laboratory ration and 1.75% NaCl solution as sole drinking fluid. Blood pressure was significantly elevated within 2 to 5 weeks and became maximal within 8 to 10 weeks (*cf.* also 5, 10, 11). The mechanism resulting in eventual hypertension is not understood but may involve sensitization by salt to circulating pressor agents(12,13) or accumulation of salt within arteriole walls(14) with subsequent reduction of lumen size. Renal lesions are ultimately observed under these conditions(11, 15). Hibbard(16) and Axelrad *et al.*(17) found that ingestion of large amounts of sodium chloride increased thyroid activity as assessed by hyperplasia, histological changes characteristic of hypersecretion and increased radioactive iodine uptake. Increase in thyroid activity may play a role in development of hypertension under these conditions, and the ameliorating effect of PTU would have a logical explanation. This drug also prevented

TABLE I. Organ/Body Weight Ratios of Rats in Exp. 1.

Treatment	No. of rats	Mean body wt (g)	Organ/body wt ratio (mg/100 g body wt)							
			Heart	Kidneys	Testes	S. vesicles	Prostate	Thyroid	Thymus	Adrenals
Chow + 1.75% NaCl sol.	7	342	441.9 ± 35.5*	1013.2 ± 43.8	834.4 ± 42.6	122.0 ± 16.2	90.4 ± 10.8	6.3 ± .6	51.4 ± 18.6	13.8 ± 1.1
			<.01†	<.01	<.01	.26	.23	<.01	.81	.34
PTU + 1.75% NaCl sol.	8	352	292.4 ± 7.9	810.4 ± 33.5	1002.6 ± 32.8	99.1 ± 10.5	112.0 ± 13.1	30.2 ± 2.1	46.5 ± 5.6	12.6 ± .5

* ± 1 stand. error of mean.
† Probability value.

cardiac and renal hypertrophy observed in non-PTU-treated controls. These results support earlier observations in which PTU treatment generally, although not always, prevented cardiac hypertrophy accompanying bilateral renal encapsulation with molded latex envelopes(2).

PTU-administration prevented growth in male and caused weight loss in female rats. In addition, testicular weight ratio increased significantly. Both effects have been reported earlier(1,2) while the latter has also been reported for thiouracil(18). Testicular effect of PTU may be due to increased gonadotrophin secretion by anterior pituitary gland or increased sensitivity of testicular tissue to normal gonadotrophin levels. Others observed increased testicular weight responses to exogenous gonadotrophin administered to thiouracil-treated rats(18) and increased compensatory hypertrophy upon removal of one testis of propylthiouracil-treated rats(19). These observations support the idea that sensitivity of testicular tissue to gonadotrophin increases. If one assumes increased pituitary secretion of gonadotrophin under these conditions, then testicular response may be due to increased secretion of FSH and not a combination of FSH and ICSH (LH), since evidence for increased secretion of testicular hormone was not observed. For example, prostate and seminal vesicle size were normal and kidney size reduced (Table I). Renotrophic effect of testicular hormone was absent(20). If ovarian weight ratio is a measure of FSH secretion, PTU-treated female rats in Exp. 2 showed evidence of decreased FSH secretion rather than an increase. No complete explanation can be given for the difference in gonadal response to PTU between the 2 sexes. Similar unpublished results have been observed previously for surgically thyroidectomized female rats whose kidneys were encapsulated with latex envelopes.

Weight ratios of adrenal and thymus glands in both male and female rats following PTU treatment are well within the range of normal, untreated rats kept under our laboratory conditions(2). These gross measurements suggest that adrenal function was unaltered either by salt treatment or by treatment with both salt and PTU. Furthermore, it has been shown that salt hypertension develops in absence of adrenal glands(21), and is maintained when adrenal glands are removed after hypertension has developed(22). These results suggest that adrenal glands do not play an active role either in development or maintenance of this type of hypertension.

While the anti-thyroid drug, propylthiouracil, prevents development of salt hypertension, the mechanism by which hypertension is prevented is unknown. Whether the results are due to direct effect of this drug on thyroid gland; to indirect effects of lack of thyroid hormone or to extra-thyroidal effects cannot be stated. However, daily intake of NaCl solution by PTU-treated rats was at least the same as that of control group. Other unpublished studies have shown that spontaneous salt intake of PTU-treated rats is considerably greater than that of normal rats. Studies are being carried out to determine effect of PTU on sodium balance since a natriuretic effect of this drug could account, at least in part, for its anti-hypertensive effect. Other studies are designed to determine whether cardiac output is reduced by PTU administration.

Summary. Dietary administration to rats of the anti-thyroid drug, propylthiouracil, prevented development of hypertension accompanying administration of hypertonic (1.75%) NaCl solution as sole drinking fluid. Propylthiouracil administration prevented not only the expected rise in systolic blood pressure but also cardiac and renal hypertrophy. Although the results suggest the possibility that thyroid gland may play a role in salt hypertension, they could be accounted for by extra-thyroidal effects of the drug.

It is a pleasure to acknowledge technical assistance of W. Pickels.

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Received June 29, 1959. P.S.E.B.M., 1959, v102.

Acid and Glucose-6-Phosphatase Activity of Pancreatic B Cells after Cortisone and Sulfonylureas.* (25227)

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In previous work(1,2) glucose-6-phosphatase (G-6-Pase) was demonstrated histochemically in mammalian pancreatic B cells. It was also found, contrary to other reports (3,4), that non-specific acid (B-glycero and alpha napthyl) phosphatase (Ac-Pase) was present throughout pancreatic islets as well as in acinar cells. The function of these enzymes in pancreatic hormonal functions is unknown. However, glucose-6-phosphate (G-6-P) available for B cell metabolism may determine insulin output. G-6-P would vary with blood glucose concentration and G-6-Pase activity. On the other hand, Ac-Pase may play a role in protein synthesis(4), specifically in production of pancreatic endocrine and exocrine secretions. Increased insulin output from B cells as indicated by diminution of pancreatic B cell granulation occurs in cortisone-induced experimental diabetes(7) and after administration of hypoglycemic sulfonylureas(8,9). In the former instance, this is mediated by hyperglycemia(5,6,7,10). whereas sulfonylureas have a direct effect on B cells(8,9). A study of G-6-Pase and Ac-Pase content of B cells, under these conditions, may be helpful in assessing previous hypotheses regarding control of B cell secretory activity, and in explaining the pancreatic action of sulfonylureas.

Material and methods. Twenty-four New Zealand white rabbits weighing 2.5 to 3.5 kg were divided into 3 groups: Group I (6 normal rabbits) served as controls. Group II (12 animals) received 5 mg/kg cortisone acetate intramuscularly once daily for 10 to 21 days. Seven developed moderately severe diabetes and were sacrificed from 10 to 21 days. Group III (6 animals) received 125 mg/kg chlorpropamide intramuscularly twice daily and were sacrificed after 8 days. All animals were killed with Nembutal. Pancreas was removed immediately and frozen in petroleum ether kept at –70° by a bath of alcohol and dry ice(11). The frozen tissue was dried with filter paper and stored in stoppered tubes in the deep freeze at –20°C. Cryostat sections at 5 to 10 μ were affixed to slides and stained immediately or placed in neutral 6% calcium formol at –4° for 10 minutes prior to staining. Additional blocks of pancreas were placed in Zenker-formol solution and processed as previously described(12). To demonstrate G-6-Pase activity, the previously described technics(13,14) were modified(1). Slides were incubated for periods of 10 to 30 minutes in a mixture consisting of 2 cc of 0.5% solution of potassium glucose-6-phosphate, 20 cc of 0.2% tris maleate buffer, pH 6.7, 3 cc of 2% lead nitrate and 7 cc of distilled water. Control sections were incubated without substrate as well as after iodine treatment. Ac-Pase was demonstrated by Gomori's method(15,16) utilizing B-glycero phosphate as substrate at pH 5.2 with acetate buffer substituted for tris maleate buffer and incubation times of 30 to 60 minutes. The Zenker fixed tissue was stained by the periodic acid Schiff trichrome method(12) and a modified aldehyde fuchsin (17) trichrome method.

Results. G-6-Pase activity in normal rabbit pancreas was confined to B cell cytoplasm which frequently appeared to be more heavily stained in the perinuclear area(1,2). Areas of islets presumably occupied by A and D cells showed no precipitate. There was only minimal diffuse staining of the exocrine parenchyma (compare Fig. 1 and Fig. 2). Ac-Pase activity was found throughout the rabbit pancreas, but was greatest as secretory poles of acinar cells and at vascular poles of islet cells (Fig. 3). Morphologic alterations in the pancreas after cortisone treatment (Fig. 4) included marked and frequently complete degranulation of B cells as well as glycogenic vacuolization of duct epithelium and frequently also of B cells(7,10,18). In animals

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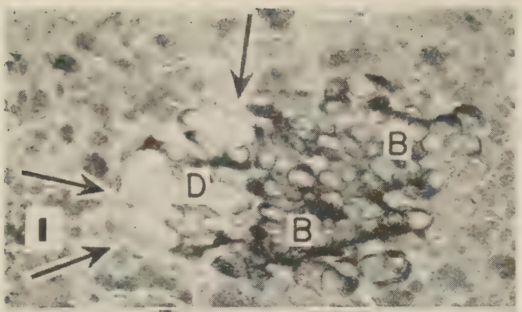
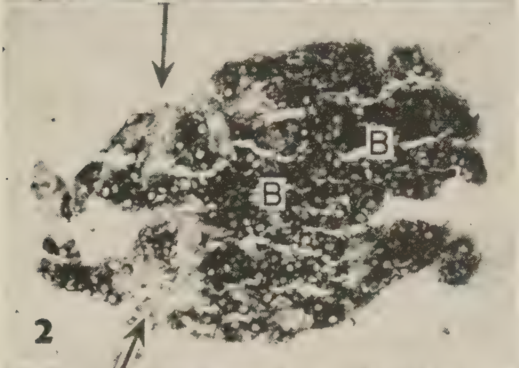
* Supported by Grant from National Science Fn., Washington, D.C.





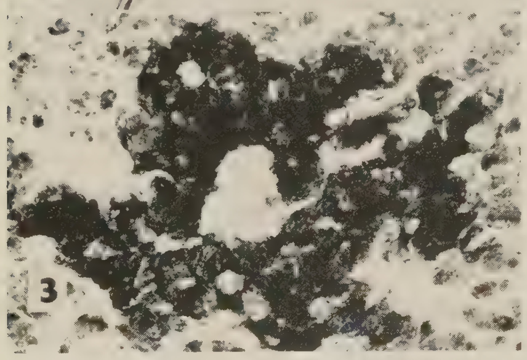


FIG. 1. Pancreas of untreated rabbit showing islet with A (arrows), B and D cells. Aldehyde Fuchsin Trichrome Stain. × 375.

FIG. 2. Pancreas of untreated rabbit showing distribution of glucose-6-phosphatase. Staining almost entirely confined to B cells. Sites of A and D cells (arrows) and acinar tissue are unstained. × 190.

FIG. 3. Pancreas of untreated rabbit showing distribution of non-specific acid phosphatase. Activity is present throughout the pancreas and most intense at vascular pole of islet cells and at secretory pole of acinar cells. × 375.

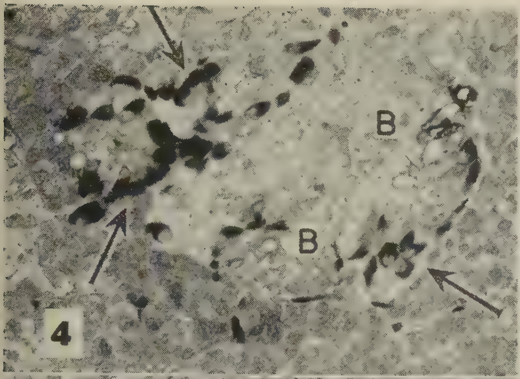
treated longest, there was diffuse proliferation of intercalated ducts, occlusion of small ductules by inspissated secretion and intraislet proliferation and dilatation of ductules. Despite these changes, no definite alteration in enzymatic staining was found, except that areas of islets occupied by proliferating duct epithelium did not show any G-6-Pase or Ac-Pase activity. After treatment with chlorpropamide as with other sulfonylureas(8,9,19,20), marked to complete B cell degranulation occurred (Fig. 5), but Ac-Pase activity was similar to normal. On the other hand, intensity of G-6-Pase staining at 10 minutes incubation was considerably less than in normal controls (Fig. 6), but this difference became less apparent after longer incubation.

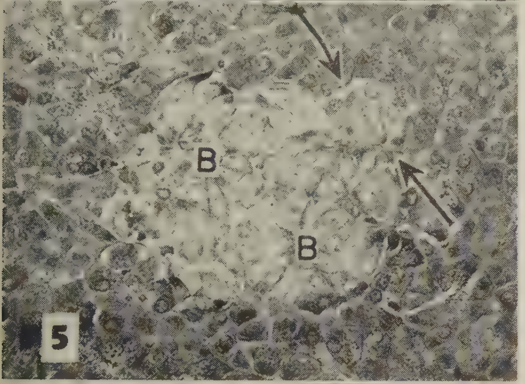
Discussion. Hyperfunctioning rabbit pancreatic B cells degranulated by cortisone-induced hyperglycemia or by sulfonylurea administration showed no grossly visible changes in Ac-Pase staining. If this enzyme is important for protein synthesis in the B cell, an increase in its activity might have been expected. However, it must be remembered that histochemical methods give rough estimates of intracellular enzyme activity.

Lack of alteration of B cell G-6-Pase after cortisone and diminution in activity after sulfonylurea are consistent with the postulated role of this enzyme in controlling insulin output. High blood sugar levels after cortisone treatment would make increased G-6-P available for B cell metabolism despite intact G-6-Pase activity and thereby increase insulin output. On the other hand, diminution in G-6-Pase activity after sulfonylurea would accomplish similar results despite lowered blood sugar level.

Biochemical studies in liver homogenates have shown a slight(21) or marked(22,23,24) increase in G-6-Pase activity after cortisone and a diminution after chronic sulfonylurea administration(25). On the other hand, both *in vivo* and *in vitro* experiments have failed to demonstrate an acute effect of the sulfonylureas on liver G-6-Pase activity(25,26), suggesting that diminished hepatic G-6-Pase activity after chronic sulfonylurea administration is an adaptive response similar to that observed after insulin administration and not due to a direct action of the drug on this enzyme. This alternative hypothesis could also apply to the reduced B cell G-6-Pase observed in the present study after sulfonylurea administration.

Summary. Glucose-6-phosphatase (G-6-Pase) and acid phosphatase (Ac-Pase) activi-





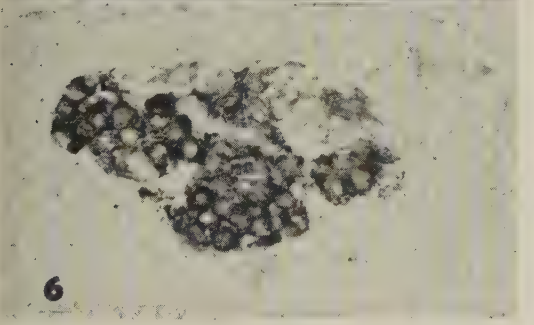


FIG. 4. Pancreas of cortisone treated rabbit showing islet with degranulated and partly vacuolated B cells and intact A cells (arrows). Aldehyde Fuchsin Trichrome Stain. × 375.

FIG. 5. Pancreas of chlorpropamide treated rabbit showing islet with degranulated B cells and intact A cells (arrows). Aldehyde Fuchsin Trichrome Stain. × 375.

FIG. 6. Pancreas of same rabbit as in Fig. 5, showing islet with reduced glucose-6-phosphatase activity. × 375.

ties have been followed histochemically in hyperfunctioning rabbit pancreatic B cells in cortisone diabetes and after administration of hypoglycemic sulfonylureas. Cortisone diabetic rabbit pancreas with B cell degranulation and glycogenic vacuolization of ductules and B cells showed no definite changes in B cell Ac-Pase or G-6-Pase activity. Hypoglycemic sulfonylureas administered over period adequate to degranulate B cells, seemed to diminish their G-6-Pase content without effecting Ac-Pase. Although B cell degranulation was, in each of these instances, indicative of increased insulin output, lack of alteration of histochemically demonstrable B cell Ac-Pase activity was not considered to be at variance with the view that this enzyme may be important for insulin synthesis. Inhibition of B cell G-6-Pase activity by chronic sulfonylurea administration could be the mechanism of pancreatic action of these drugs. However, the alternative explanation that reduced G-6-Pase activity was a secondary adaptive response cannot be entirely excluded.

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Received July 1, 1959. P.S.E.B.M., 1959, v102.

Purine Antagonists and Growth of Tetrahymena.* (25228)

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The demonstration that a purine analog interferes with the metabolism of acetate and with sterol synthesis in *Tetrahymena*(1) led to a reexamination of the effects of other structural analogs of purines. These compounds may be divided into groups on the basis of the nature of structural changes embodied in them, nature of substances bringing about reversal and whether or not the inhibitors are able to bring about death of the cultures. These features can be shown to have a certain degree of correlation to one another.

Material and methods. Strain W of *Tetrahymena pyriformis* was grown in medium A (2) containing Tween 80 at a concentration of 10 mg/ml. From this medium acetate and adenylic acid were omitted as indicated. In certain experiments β-sitosterol or adenine was added. Conditions of growth and incubation were as previously described(2). The purine analogs 8-azaguanine (8AG), 8-azaadenine (8AA), 6-methylpurine (6MeP), 6-chloropurine (6ClP) and purine (Pu) were commercial products. One sample of 6-methoxypurine (6MeOP) was prepared in the Biological Laboratory at Amherst College and another obtained commercially. The sample of 6-methyl-1-deazapurine (6MeDAP) was also prepared in the Amherst College laboratory. Through the kindness of Dr. K. Pfister of Merck and Co. we obtained a sample of 2-azaadenine (2AA).

Results. Table I shows the response of *Tetrahymena* to the various inhibitors in the basal medium and also with addition of acetate, β-sitosterol or adenine or combinations of these substances. The first 3 inhibitors represent the group of analogs in which changes in ring structure have been made and all (Figures in parentheses) are most readily reversed by adenine. Addition of acetate, sterol or both have relatively little influence on the inhibition. The next group of 4 analogs differs from adenine only in the nature of the substituent replacing the amino group in the 6 position. Two of these compounds, 6MeP and 6ClP are reversed best by sterol and synergistic reversing effects are shown by combinations of adenine with sterol or acetate. The other 2 compounds, Pu and 6MeOP, are almost equally well reversed by either adenine or sterol and the effects of combinations of material are not so striking. The last analog (6MeDAP) has changes both in the ring and in the substituent group. It resembles the first group of analogs in its reversal by adenine. With regard to 1-deazaadenine (DAA) it can only be said that it is reversed by adenine(3). There are no data on the effects of the other compounds considered here.

Certain of these purine analogs have another peculiarity in common. They permit normal growth of the organisms up to a point. The organisms then lose motility, settle to the bottom of the tubes and autolyze. This process releases fat droplets, glycogen granules and other particulate material into the medium, thus making it more, rather than

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* Supported by research grants (A-1005 and CY-2924) from the National Institutes of Health, U. S. Public Health Service and a grant (CH-27) from the American Cancer Society.

TABLE I. Inhibition of *Tetrahymena* by Purine Analogs.

Inhibitor	8-azaguanine				2-azaadenine				8-azaadenine				Purine			
	γ/ml															
Additions	.2	.4	.8	2	5	10	20	50	50	100	200	500	100	200	500	1000
	% inhibition															
None	17	44	60	70	33	46	54	90	37	44	50	89	31	35	44	60
Acetate, 1 mg/ml	33	49	52	58	33	46	62	90	32	38	50	90	30	33	39	48
Sterol, 25 γ/ml	22	43	51	55	37	49	57	70	39	48	53	71	39	41	43	50
Adenine, 20 γ/ml	11	22	(25)	30	13	17	(37)	89	3.5	10	(19)	93	19	26	(39)	46
+ acetate	11	20	28	36	7	21	40	88	2	6	17	87	11	22	30	41
+ sterol	17	25	28	35	13	25	35	81	4	9	16	68	18	23	28	32
+ acet.	17	24	30	36	10	20	32	75	1	3.5	8.5	47	13	19	19	27

Inhibitor	6-methylpurine				6-chloropurine				6-methoxypurine				1-deaza-6-methylpurine			
	γ/ml															
Additions	.2	.4	.8	2	50	100	200	500	100	200	500	1000	100	200	500	1000
	% inhibition															
None	28	42	71	93	1	12	46	93	0	4	56	97	6	24	53	93
Acetate	31	36	51	76	0	0	17	92	0	4	41	96	8	22	52	91
Sterol	26	34	(44)	79	2	8	(17)	44	0	0	34	96	18	34	60	93
Adenine	10	11	68	95	4	10	33	84	2	3	(31)	95	4	5	(10)	86
+ acetate	8	10	25	71	2	5	13	74	1	0	24	93	4	5	11	85
+ sterol	3	5	14	60	3	10	21	64	0	0	15	92	4	6	12	86
+ acet.	4	7	11	26	1	7	16	74	0	0	12	86	2	3	9	86

less, turbid. Table II shows the various properties of the analogs and their correlation with structural changes. While reversibility by adenine appears to be correlated with changes in ring structure and reversibility by sterols with the substituent in the 6 position, ability to cause death of the cultures is not clearly related to one type of structural change rather than the other.

An apparently obvious omission from this list of analogs is 6-mercaptopurine. This compound is inactive as a growth inhibitor for *Tetrahymena*.

Discussion. The fact that acetate and/or sterols are active to some degree in reversal of all of a series of 8 purine analogs and in certain cases are the most active compounds in this respect, indicates an involvement of purines at some point in synthesis of sterols and other lipids. This point does not appear to be in activation of acetate to form acetyl adenylate (prepared by M. R. Heinrich by published methods(4,5,6)), since the latter was no more active than an equimolar mixture of acetate and adenosine-5′-phosphate in reversing inhibition by 6MeP. The data presented by Wright(7), however, suggest that a certain type of ribonucleic acid or polyribonucleotide is necessary for sterol synthesis and the explanation of the results presented above may lie in a requirement for synthesis of such polynucleotides from precursors such as adenine.

TABLE II. Characteristics of Certain Purine Analogs.

	Structural change		Reversing compound		
Analog	Ring	Substituent	Adenine	Sterol	Death
8AG	+	—	+	—	—
2AA	+	—	+	—	—
8AA	+	—	+	—	—
DAA	+	—	+	?	+
6MeP	—	+	—	+	—
6ClP	—	+	—	+	—
Pu	—	+	+	+	—
6MeOP	—	+	+	+	+
6MeDAP	+	+	+	—	+

It is obvious from the large variation in amounts of the analogs required to produce equal degrees of inhibition that there is also a large variation in affinity of these inhibitors for the enzymes metabolizing purines. It would also appear that there is a differential affinity among the various enzymes such that different analogs are not equally reversible by all of the active compounds.

Since *Tetrahymena* is not inhibited by 6-mercaptopurine, this interesting compound could not be included in this series. With *Escherichia coli* it has been shown, however, that 6-mercaptopurine decreases incorporation of radioactive acetate(8). This would indicate that the effect of purine analogs on acetate metabolism is not confined to *Tetrahymena*. Also 6-mercaptopurine may act in much the same way as the analogs described above.

No explanation is yet available for the death of cultures containing certain of the purine analogs. It cannot be accounted for on the basis of accumulation of acid as is the case in cultures deficient in either thioctic acid (9) or thiamine(10). Among other compounds which cause "suicide" of *Tetrahymena* are protopine and α-phenylbutyric acid, an analog of acetate (unpublished observations). So far it has not been possible to reverse the inhibition caused by protopine, but all the other "suicidal" compounds or conditions are in some way involved in acetate metabolism.

Summary. Eight purine analogs were compared as to their effects on growth of *Tetrahymena*. A certain degree of correlation was found between type of structural change present in the analog and ability of adenine, acetate, sterol or combinations of these compounds to reverse the inhibition. Analogs which cause "suicide" of cultures do not fit the pattern of other inhibitors.

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Received July 1, 1959. P.S.E.B.M., 1959, v102.

An Activator of Plasminogen Produced by Cell Culture. (25229)

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A simplified scheme, modified from Astrup (1) for activation of plasminogen by "activators" or protease, and for demonstration of such activation by clot lysis or casein hydrolysis is shown in Fig. 1. There are many activators of plasminogen. Those of note are streptokinase, particulate "tissue activators," potassium thiocyanate extracted "tissue activators," microsome fractions of animal tissues, urokinase, human milk, trypsin, and chloroform. In addition, spontaneous activation may occur(1). This study describes materials released by metabolizing cell cultures which activate plasminogen(2) or which are proteolytic in the absence of plasminogen(3,4).

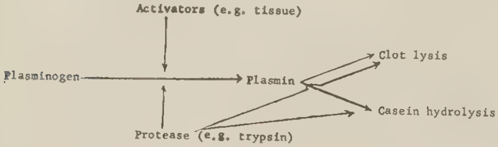

FIG. 1.

Materials and methods. Clarified supernatant medium from cell cultures was used as a source of plasminogen activator and of caseinolytic protease. The supernatant medium was prepared by feeding Medium 199(5) to cell cultures which had been previously washed 4 times with balanced salt solution (b.s.s.). After incubation for 96 hours at 36°C it was decanted and clarified by low speed centrifugation. Supernatant medium

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TABLE I. Activator of Plasminogen, and Protease from Cell Cultures.[*]

Exp.	Source of plasminogen	Activator	Substrate	Result
1	HS, PP	KB cell culture	SM, BC	+
2 a	HS, PP	KB supernatant	"	+
b	None	" "	"	0
c	HS, PP	None	"	0
d	None	KB supernatant + SK	"	0
3 a	None	MK supernatant	"	+
b	HS, PP	" "	"	++++

[*] Abbreviations are as follows: HS = human serum; PP = purified plasminogen; SM = skim milk; BC = bovine clot; SK = streptokinase (Varidase® Lederle). + = Proteolysis; 0 = no proteolysis; ++++ = accelerated proteolysis.

was assayed for activator of plasminogen by Christensen's method(6) employing the lysis of bovine fibrin clots and by a method employing the hydrolysis of casein(7) as indicated by the clearing of the opalescence of autoclaved skim milk(2). Duplicate serial 2-fold dilutions of supernatant medium in 0.2 ml amounts were made in Veronal® buffer solution (VBS), pH 8.2, ionic strength 0.05. To one dilution series, used to assay for activator, 0.1 ml of a 1:30 dilution of pooled human serum (HS) was added as the source of plasminogen, and to the other dilution series used to assay for protease, 0.1 ml of VBS was added. Then 0.1 ml of a 1:2 dilution of autoclaved skim milk (SM) was added as substrate to both series. A serum control containing only HS, VBS, and SM substrate served to control for spontaneous activation of plasminogen. Plates of clear vinyl plastic cups, Disposotrays® model 96 CV[†] were used instead of test tubes. Cups were covered with 0.6 ml sterile heavy mineral oil, incubated at 36°C and the clearing of opalescence of SM observed daily. A preparation of supernatant medium was considered to have a titer of activator or protease in units equal to the reciprocal of the highest dilution which caused clearing at 96 hours incubation.

Results. Activator of plasminogen and protease produced by cell cultures. KB cell culture (KB), derived from a human epidermoid carcinoma(8), caused clearing of skim milk or clot lysis if a source of plasminogen were present (Table I, Exp. 1). The continued presence of cells was not essential for this reaction since the supernatant medium, free of cells (Table I, Exp. 2), caused proteolysis in the presence of plasminogen. Streptokinase (Varidase,® Lederle Laboratories) plus the supernatant medium did not produce proteolysis, *i.e.*, plasminogen was not present in the supernatant. These observations indicated release of an activator of plasminogen from KB cell culture. Supernatant from primary monkey kidney cell culture (MK)(9) (Table I, Exp. 3) caused proteolysis in absence of plasminogen and accelerated proteolysis when tested in presence of plasminogen. These observations indicate the presence of both protease and an activator of plasminogen in the supernatant medium from MK.

Characteristics of activator from cell culture. To determine the effect of various ratios of concentration of activator, *i.e.*, supernatant medium from KB cell culture, and of HS on the time required to clear SM, one was diluted and incubated with dilutions of the other (box titration). Increased concentrations of activator or HS up to an optimum (final concentration of 0.8% HS and 12.5% KB supernatant) shortened the time of clearing. Further increase delayed time of clearing. This delay may be attributed to inhibitors of proteolysis which have been described in tissue(1) and human serum(10). Human euglobulin(11) and purified plasminogen, "Pro-Actase"®, kindly supplied by the Ortho Co., showed less inhibition at the higher concentrations than did HS. Increased concentrtaions of SM substrate (5-20%) delayed time of clearing.

Evidence for hydrolysis of casein was the rise in optical density of the trichloroacetic acid filtrate at 275 mμ when SM substrate was incubated with activator from MK supernatant and euglobulin. This corresponded in time with clearing of the opalescence of SM. Identical evidence was obtained when KB supernatant or streptokinase-streptodornase (Varidase®, Lederle Laboratories) was used as activator for human euglobulin or human, chicken, rabbit and monkey serums. KB supernatant which assayed as 64 units of activator by milk clearing, had activity equal to

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[†] Obtained from Joseph E. Frankle Co., Phila. Pa.

2 units of streptokinase when measured by Christensen's method(6) using clot lysis. Thus, the clot lysis method was not sufficiently sensitive for assay of most lots of unconcentrated supernatant medium.

The activator in supernatant medium from KB cell culture was non-dialyzable, stable to 3 cycles of freezing and thawing, active at pH 7 and pH 8.5, stable on storage at 4°C or –20°C for at least 6 weeks, but showed an inconstant slight drop in titer after 1 week at 36°C or 20°C. Complete inactivation occurred after 10 minutes at 80°C or 3 hours at 56°C, and after filtration through very fine fritted glass or Seitz S3 filters. Activity was sedimented at 18,000 g for 90 minutes (microsome size). Activator from KB supernatant after treatment with 1M KSCN(12), showed no change of titer and showed only a minimal loss of titer after filtration. The activator is thus made soluble and filterable by treatment with KSCN, which is a property described for "tissue activator."(12).

Source of activator produced by KB cell culture. Experiments were designed to determine if activator released from cell culture represents portions of an intracellular store of activator or represents materials being continually produced by metabolizing cells. The supernatant medium was removed daily from cell cultures, the cells washed, and the cells fed with fresh medium. The decanted medium was assayed for activator using SM as substrate. Sixteen units of activator were released daily from a 32 oz. cell culture bottle containing approximately 3 x 10^7 KB cells with 50 ml of Eagle's medium or Medium 199. The release of activator remained constant for 4 days and diminished during the next 3 days as the cell sheet degenerated. Release of activator from cell cultures in lactalbumin hydrolysate medium(13) was diminished and variable. To determine whether activator which was continually released from KB cells came from intracellular stores or was continually produced, intracellular activator content was measured as follows. KB cells were scraped from 32 ounce cell culture bottles and washed 4 times in 30 ml of b.s.s. by low speed centrifugation. After washing, the cells were resuspended in sufficient VBS to give a concentration of 10^7 cells/ml. Aliquots of cells in suspension were disrupted by 3 cycles of freezing and thawing or by sonic oscillation in a 250 watt, 10 kilocycle, Raytheon sonic oscillator at maximum input for 10 minutes. Samples of intact and disrupted cells were extracted with KSCN. The resulting 4 preparations of disrupted cells were assayed for activator and protease using SM as substrate. In no instance were more than 2 units of activator detected in any of these preparations although as many as 3 x 10^7 cells were used. Assays for inhibitors of proteolysis in these preparations indicated that the inhibitor content of KB cells was insufficient to mask appreciable amounts of intracellular activator. The data testifies for production of activator by metabolizing cells rather than for release of active intracellular material by cell breakdown.

Comparison of MK supernatant with KB supernatant. Serum-free Medium 199 from MK was found to contain 32 units of protease when tested without serum and 512 units of activator when tested in the presence of serum, using SM as substrate. The activity as caseinolytic protease was minimally diminished after filtration through very fine glass while the activity as activator of plasminogen was diminished to a greater extent after filtration. Thus, MK supernatant consistently had an activator: protease ratio of 4:1 to 8:1 after filtration, regardless of how great this ratio was prior to filtration. As previously stated, KB supernatant preparations with as much as 512 units of activator showed activity only as activator of plasminogen and the activity was not filterable. An interpretation might be that both KB and MK cells release a microsome-size activator. In addition MK cells release a filterable trypsin-like protease which can activate plasminogen as demonstrated by the 4 to 8 fold increase of titer in the presence of serum.

Summary. Previous investigators observed clot lysis by tissue grown in plasma clots(14, 15). Our results indicate that an "activator" of plasminogen is released from KB cell cultures. Supernatant from MK cell cultures activated plasminogen and was also proteolytic in the absence of plasminogen. These cell cultures had a third property, inhibition of proteolysis. The activator described here resembles "tissue activator" in that it is not proteolytic in the absence of serum and can be dissolved by KSCN. It also resembles activator from excised rat lung(16) in that it is the size of microsomes. The interpretation that release of activator from cell culture is a metabolic process and not from cell breakdown seems warranted since only a small fraction of the activity found in the supernatant can be extracted from the cells.

The technical assistance of Richard J. Low, Alice M. Gochenour, and Beatrice L. Burch is gratefully acknowledged. The authors thank Drs. Hilton B. Levy, Caspar W. Hiatt and Raphael N. Shulman for suggestions and for reviewing this manuscript.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Enzymatic Determination of *in vitro* Lysis of Leukocytes by Tuberculin.
(25230)

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In vitro lysis of leukocytes by tuberculin, described by Favour(1), generally is determined by microscopic counts of leukocytes before and after incubation in presence and absence of tuberculin. The sensitivity of this method depends on the accuracy of cell counts. O'Neill and Favour(2) reviewed the factors affecting leukocyte counts and concluded that the mechanical errors inherent in the method can be controlled so that an overall error of less than 10% may be obtained. In practice, however, a cytolysis of less than 10% is not considered significant(3,4). Another limiting factor in the method is the time involved in performing large numbers of cell counts. For these reasons, we have been interested in finding a method for determining leukocyte lysis which is more rapid and more sensitive than microscopic cell counts. Kerby (5) described a method to demonstrate leukocyte injury and lysis, based on the fact that certain cellular components are released into the surrounding medium when leukocytes are damaged. Using release of a lysozyme-like enzyme as a measure of cell damage, Kerby demonstrated a harmful effect of certain bacterial derivatives on human leukocytes(6), and later showed that ACTH and cortisone therapy protected human leukocytes against injury by piromen(7). This paper describes our attempts to use release of this intracellular enzyme as a measure of lysis of leukocytes by tuberculin.

Materials and methods. The technic for assay of lysozyme-like enzyme was similar to that described by Kerby(5). However, it was necessary to use M/15 phosphate buffer at pH 7.0 in place of pH 6.2 buffer because the latter caused precipitation in some samples of guinea pig plasma. A 200 mg % suspension of *Micrococcus lysodeikticus* (Difco) in the phosphate buffer was used as substrate for purified egg-white lysozyme (Difco) to prepare a standard curve in the range of enzyme found in plasma of normal and tuberculin-positive guinea pigs(8). One-tenth ml aliquots of various enzyme concentrations were diluted in 0.9 ml of phosphate buffer and 1.5 ml of substrate. Optical density of suspensions was read with an electrophotometer (Fisher) at 525 mμ before and after 20 minutes incubation at 37°C after standardizing the instrument with a buffer blank. The per cent lysis of substrate was calculated as the difference between initial and final optical density divided by initial optical density. Normal male albino guinea pigs (400 g) were sensitized to tuberculin by intramuscular injection of 5 mg of heat-killed *M. tuberculosis* (H37Rv) suspended in 1 ml of sterile paraffin oil. Cytolysis tests were performed on day of bleeding, using heparinized (0.4 mg/ml) blood samples collected by cardiac puncture from fasted animals. Four siliconized serology tubes, each containing 0.8 ml of whole blood, were prepared for each sample. To 2 tubes was added 0.2 ml of pyrogen-free saline (Baxter) containing 10 μg of PPD (Sharp and Dohme); to the 2 remaining tubes was added 0.2 ml of saline. Tubes were sealed with silicone stoppers and rotated at 37°C on roller drum (5 rpm) for 2 hours. After rotation, samples were removed for total leukocyte counts. Both chambers of a standard hemocytometer (AO Spencer) were counted for each sample. The per cent PPD lysis for each sample was calculated as the difference between average cell counts of duplicate saline and PPD tubes divided by average cell count of the saline tubes. After samples were removed for leukocyte counts, all tubes were centrifuged at 1,000 x g for 20 minutes and assays for lysozyme-like enzyme were performed on 0.1 ml aliquots of the supernatant fluid. To each sample was added phosphate buffer and substrate as described above. Per cent lysis of substrate was converted to lysozyme equivalents by reference to standard curve. In addition, the lysozyme Δ was calculated for each sample by subtracting the average per cent lysis of substrate by saline tubes from that obtained with PPD tubes. Positive values for lysozyme Δ were considered indicative of tuberculin cytolysis since the higher values of the PPD tubes were assumed due to increased enzyme levels originating from lysed leukocytes. Preliminary tests showed that PPD in the concentration used had no effect on enzyme or substrate.

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* Material presented forms part of thesis to George Washington University, Washington, D. C., in partial fulfillment of requirements for degree of Doctor of Philosophy.

Results. The results presented in Table I show release of lysozyme-like enzyme from leukocytes following exposure to tuberculin *in vitro*. Each value represents the mean of duplicate samples from 4 sensitized animals, tested for cytolysis from 7 to 12 days following injection of heat-killed tubercle bacilli. By the leukocyte count method, each group showed significant cytolysis as indicated by the fact that 10% or more of the cells were lysed by PPD. Actual number of cells lysed was calculated as the difference between total number of leukocytes of the saline and PPD tubes and is shown as lysed cell contents/tube. The lysozyme equivalents represent enzyme originating from the lysed leukocytes in excess of that in plasma of surrounding medium. The concentration of enzyme in tubes containing PPD was significantly higher (P = 0.025) than that in saline tubes. The content of enzyme in guinea pig peripheral blood leukocytes was calculated from the experimental results.

The application of the lysozyme assay method for detecting leukocyte lysis is further illustrated in Fig. 1 which shows development of leukocytic susceptibility to tuberculin following injection of guinea pigs with heat-killed tubercle bacilli as measured by total leukocyte counts and by the lysozyme assay method. Each point represents the mean of duplicate samples from 4 animals. Statistically significant cytolysis (P = 0.025), as reflected by positive values of the lysozyme Δ, occurred at 10 and 12 days. By the leukocyte count method, significant cytolysis also occurred at 10 and 12 days.

Discussion. Our results indicate that release of a lysozyme-like enzyme is an accep-

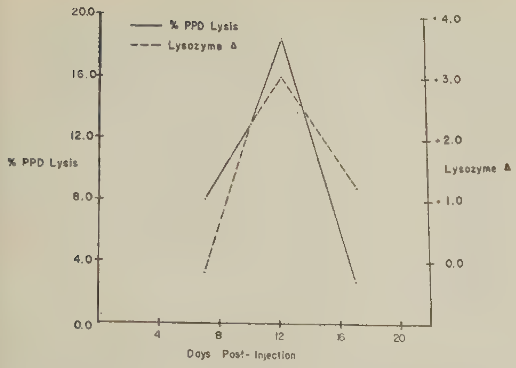

FIG. 1. Leukocyte cytolysis following injection of guinea pigs with heat-killed tubercle bacilli.

table substitute for microscopic cell counts in measuring *in vitro* lysis of leukocytes by tuberculin. It is of interest that, in previous studies with guinea pigs using the leukocyte count method, 10% cytolysis was accepted as significant(3,4). Using the method described here and assuming that the guinea pig has a total leukocyte count of approximately 7,000/mm^3, this degree of cytolysis would indicate lysis of 560,000 leukocytes. These cells would release 0.070 µg of enzyme (Table I) which, by the lysozyme assay method, would produce a lysozyme Δ of approximately + 2.0. This value appears the minimum necessary for significance since a mean lysozyme Δ of + 0.4 with a standard error of the mean of 0.6 was obtained when leukocytes from 16 normal guinea pigs were exposed to PPD under similar conditions. These calculations are supported by the correlation between the cytolysis measured by both methods (Fig. 1). This study and several subsequent experiments have convinced us that the lysozyme assay method is an adequate measure of leukocyte lysis by tuberculin. The relative speed and simplicity of the method adds to its applicability. It seems possible that the method may be useful in other systems involving leukocyte lysis provided the reagents employed are carefully investigated for activity against enzyme and substrate. We observed that markedly hemolysed plasma and serum interfered in the assay method. Similarly, as reported by Kerby and Eadie(9), heparin in concentrations greater than 7 mg/ml was inhibitory in the system. However, this is far in excess of the 0.4 mg heparin used for anticoagulation in the present study.

TABLE I. Leukocyte Cytolysis and Release of a Lysozyme-Like Enzyme.

Group	% PPD lysis	Lysed cell contents/tube	Lysozyme equivalents	
			µg/tube	µg/10^6 cells
		× 1000		
1	10.0	660	.077	.117
2	14.7	782.56	.104	.133
3	12.9	619	.077	.124
4	18.5	814	.104	.128
Mean				.126

Kerby and Chaudhuri(10) were unsuccessful in demonstrating *in vitro* tuberculin cytolysis of leukocytes from tuberculous humans by a lysozyme assay method. However, this failure does not detract from the applicability of the method since it is possible that the cells from tuberculous subjects were not susceptible to lysis by tuberculin. Their study would have been more valuable if actual tuberculin sensitivity of the cells were ascertained by the leukocyte count method. Favour and his coworkers(11) reported that leukocytes from tuberculin hypersensitive individuals vary markedly in their sensitivity to tuberculin. The demonstration of tuberculin cytolysis of leukocytes from sensitized guinea pigs (Fig. 1) agrees with the results by Waksman(3) and closely parallels the presence of a circulating "plasma factor" responsible for the cytolysis phenomenon as reported previously from our laboratory(4).

Summary. A method based on release of an intracellular, lysozyme-like enzyme from lysed leukocytes was an acceptable substitute for microscopic cell counts in measuring tuberculin cytolysis of leukocytes from guinea pigs injected with heat-killed, human type, *M. tuberculosis*.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Calcification XXVII. Collagen Transformation under Influence of Calcium and Phosphate Ions.*† (25231)

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Earlier studies have shown that fibers of acid-soluble collagen reconstituted with chondroitin sulfate mineralize *in vitro* with formation of apatite crystals(1-4). Such fibers have the non-striated form(5,6). The collagens occurring in animal tissues generally have a periodicity of 640 Å, although in preosseous cartilage which mineralizes, no periodicity is discernible under electron microscopic examination. Glimcher, *et al.*(7), have reported that, of the various types of reconstituted collagen fibers, only those with 640 Å spacings are able to nucleate hydroxyapatite. The question arose whether non-striated collagen fibers undergo any structural changes during incubation in calcifying solution.

Methods. Solutions of acid soluble collagen in 0.04% acetic acid were prepared from rat tail tendon as previously described(4). Collagen fibers were reconstituted by mixing samples of 5 ml of the collagen solution with 0.25 ml of a 1% solution of whale tracheal chondroitin sulfate‡ in 0.04% acetic acid. Fibers were further reconstituted from the collagen solution by adding a 30% NaCl solution up to a final concentration of 1% NaCl. The precipitates obtained with chondroitin sulfate were washed with distilled water, and those obtained with NaCl were washed with a solution of 1% NaCl. The washed precipitates were employed for further studies.

The basal salt solution used in the experiments was a carbonate buffer containing in mE/L, sodium: 95.0; chloride: 80.0; bicarbonate: 22.0; and potassium: 5.0. This solution was diluted 10 times with doubly-distilled water. If needed, Na_2HPO_4 and/or $CaCl_2$ stock solutions were added to the diluted basal salt solution, resulting in concentrations of 5 mg % P and 15 mg % Ca. The pH of the solutions was adjusted to 7.3 ± 0.05, as described earlier(8). The ionic strength of the solutions employed was about 0.10.

Collagen fibers reconstituted with chondroitin sulfate were incubated in diluted basal salt solution containing calcium or phosphate or both calcium and phosphate (calcifying solution) at pH 7.3 ± 0.05 and 37°C. Time of incubation in the various solutions was 1 and 2 days. Incubation in calcifying solution was also carried out for 6 hours. At the end of incubation the fibers were washed with distilled water until, after adding a few drops of 3% $AgNO_3$ solution, no precipitation of AgCl occurred in the washing fluid. The fibers were then stored in distilled water in the refrigerator for use in electronmicroscope studies. Collagen fibers reconstituted with NaCl were used without further pretreatment.

Samples used for the electronmicroscope studies§ were cut in fine segments with surgi-

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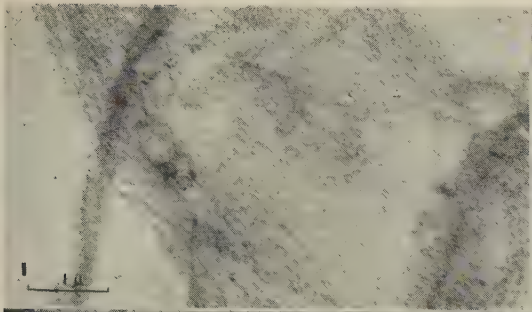
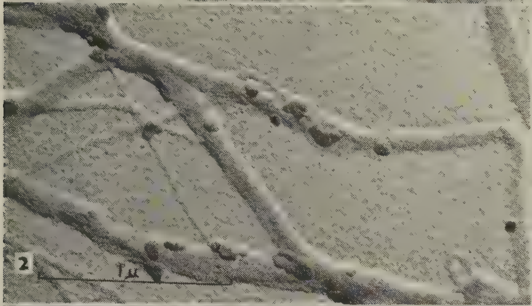
[*] Presented in part before IVth Internat. Cong. of Biochem., Vienna, Austria, Abstr.

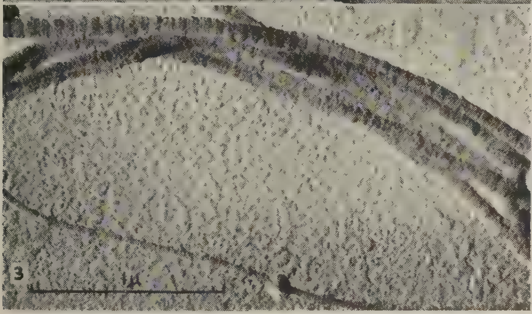
[†] This research supported in part by Air Force monitored by School of Aviation Medicine, USAF, Randolph AFB, Texas; Office of Naval Research; Nat. Inst. of Dental Research, N.I.H., P.H.S.

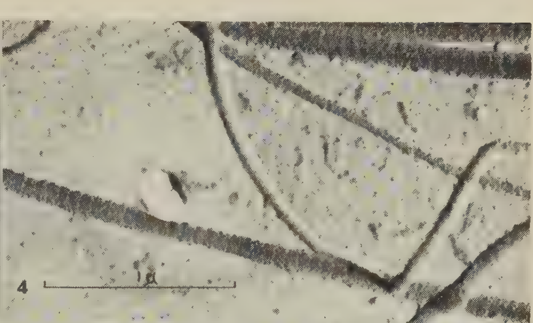
[‡] We wish to thank Prof. Erik Jorpes for this preparation of chondroitin sulfate, prepared according to method of Strandberg (*Acta Physiol. Scand.*, 1950, v21, 222) and probably consists of a mixture of chondroitin sulfate A and C.

[§] Electronmicroscopy done by Ladd Research Industries, Roslyn Heights, L. I., N. Y.









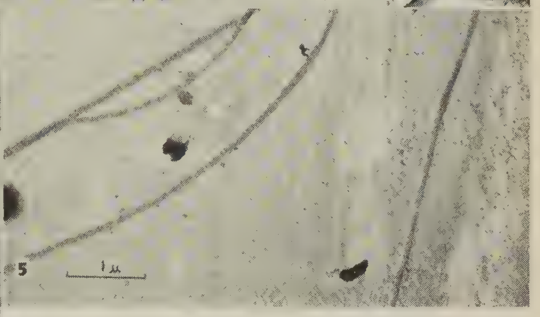


FIG. 1. Non-striated sheets of collagen fibers reconstituted with chondroitin sulfate. × 10,000. Ladd No. 264.

FIG. 2. Non-striated fibers of collagen after 2 days incubation in basal salt solution at pH 7.3 and 37°C. × 25,000. Ladd No. 280.

FIG. 3. Fibers with 640 Å spacings after 6 hr incubation in basal salt solution containing 5 mg % P and 15 mg % Ca (calcifying solution) at pH 7.3 and 37°C. × 25,000. Ladd No. 486.

FIG. 4. Fibers with 640 Å spacings after 1 day incubation in basal salt solution containing 15 mg % Ca at pH 7.3 and 37°C. × 25,000. Ladd No. 504.

FIG. 5. Fibers with 640 Å spacings after 1 day incubation in basal salt solution containing 5 mg % P at pH 7.3 and 37°C. × 10,000. Ladd No. 439.

cal scissors and dispersed in distilled water. No fixation was used. A drop of the suspension was put down on a Formvar film supported by a microscope grid. Before the specimen was completely dry, it was repeatedly washed with distilled water using a fine bore pipette. When dry the fibers were shadowed with platinum and lightly coated with evaporated carbon for stabilization under the electron beam. Unshadowed specimens were also lightly coated with carbon.

The complete experiments were repeated 3 times and the results were confirmed each time. The prepared specimens were examined in a Philips E.M.—100 B electronmicroscope and micrographs taken of representative fields. In all, some 1,100 micrographs were taken in the study.

Results. Fibers reconstituted with 1% NaCl showed very clearly the 640 Å spacings under the electronmicroscope. Collagen reconstituted with chondroitin sulfate, however, usually showed sheet-like structures without indication of spacings (Fig. 1). After treatment with basal salt solution at pH 7.3 and 37°C for 2 days some rearrangement of the sheet-like structures had occurred, giving rise to fibers of 300-1500 Å thickness, in which no indication of spacings was discernible (Fig. 2). After incubation for only 6 hours in calcifying solution (basal salt solution + 5 mg % P + 15 mg % Ca) at pH 7.3 and 37°C, virtually all sheet-like structures had transformed to the fibrous form with 640 Å spacings (Fig. 3). Treatment with basal salt solution containing either 5 mg % P or 15 mg % Ca also caused

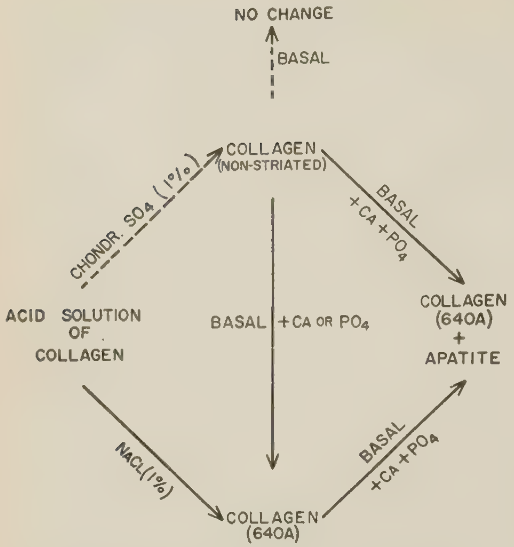


FIG. 6. Influence of calcium and phosphate on transformation of reconstituted acid-soluble collagen fibers (non-striated) to 640 Å spaced striated form.

transformation to this form (Fig. 4, 5), but in several experiments this transformation was only partial, even after 2 days. Transformation to fibers with 640 Å spacings in basal salt solution containing calcium only, occurred to a larger extent than in this solution containing phosphate only.||

Discussion. These studies show that either calcium or phosphate ions, when present in a basal salt solution at 37°C and pH 7.3, will cause rapid transformation of non-striated collagen fibers placed in this solution to the form with spacings of 640 Å. If both ions are present the transformation is more rapid and more complete. In contrast to this, basal salt solution containing neither calcium nor phosphate ions does not bring about this transformation (Fig. 6). Our finding that fibers reconstituted with 1% NaCl show predominantly 640 Å spacings confirms results obtained by other investigators(9).

That the various types of collagen fibers are interconvertible after reextraction with acid buffers has been reported(10,11). To our knowledge this is the first report on direct transformation of one type of fiber into another by specific agents.

From the results obtained in the transformation studies, one may cautiously suggest that either calcium or phosphate ions interact with a critical site in the non-striated collagen, where an electrostatic bond between positively and negatively charged groups is split, allowing the rearrangement of the patterns of aggregation of tropocollagen units to the 640 Å spaced fibers. Alternately, but less likely, 2 independent sites, one binding calcium and the other binding phosphate, might produce such rearrangement. To illustrate, electrostatic bonds within the collagen fibril which might be ruptured could be bonds between terminal amino and carboxyl groups on the tropocollagen units, or more likely, side chain bonds between the ϵ-amino groups of lysine or hydroxylysine and δ-carboxylic group of glutamic acid or the σ-carboxylic group of aspartic acid(12,13).

A further study to determine degree of specificity of calcium and phosphate ions in this transformation may shed light on the intriguing question whether the groups whose interaction with calcium and phosphate bring about transformation are the same as those responsible for formation of nuclei of hydroxyapatite crystal growth(1-4,7).

Summary. 1) Fibers of acid-soluble collagen reconstituted with chondroitin sulfate showed sheet-like structures without indication of spacings. After treatment for 2 days with basal salt solution at pH 7.3 and 37°C, containing neither calcium nor phosphate ions, non-striated fibers were formed of 300-1500 Å thickness. If either calcium or phosphate ions were present partial transformation to the form with 640 Å spacings took place. If both of these ions were present this transformation occurred more rapidly and more completely. 2) These findings suggest that calcium and phosphate ions interact with sites on the collagen fiber where the breaking of electrostatic bonds catalyzes transformation to the 640 Å spaced fiber.

|| Crystals seen in photographs originate from salts of basal salt solution, which were not completely washed out during preparation of samples for electronmicroscopy.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Influence of Bile Acids on Cholesterol Metabolism in the Mouse.* (25232)

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Cholic and dehydrocholic acids alter rates of cholesterol synthesis and mobilization in rat liver and adrenal(1,2,3). Since the results of these and other investigations(4,5) have implicated the bile acids in accumulation of dietary cholesterol and in homeostatic control of cholesterol metabolism; cholic, hyodeoxycholic, deoxycholic and lithocholic acids, were selected for this study of cholesterol metabolism in mouse tissues.

Procedure. Seventy Webster strain female albino mice, weighing 18-20 g, were placed on synthetic basal cholesterol free diet (CFD) (6) for 2 weeks, then divided into 7 equal groups. Groups A and F were continued on CFD; Group B received CFD plus 0.5% cholic acid; Groups C and G, CFD plus 0.5% hyodeoxycholic acid; Group D, CFD plus 0.5% deoxycholic acid; and Group E, CFD plus 0.5% lithocholic acid. After receiving these diets *ad lib.* for 3 weeks, all animals were given an intraperitoneal injection of 5 μc sodium acetate-1-C^{14} in saline. Mice in Groups A, B, C, D and E were sacrificed one hour after injection; those in groups F and G, 15 minutes after injection. Liver, small intestine and kidneys were removed for determination of total cholesterol, as previously described (7), and cholesterol-x-C^{14}. The latter was isolated as the digitonide and counted in a Windowless Flow Counter(1).

Results. The effects of bile acids on cholesterol levels and synthesis rates in mouse liver, small intestine, and kidney are presented in Tables I and II.

Cholic acid produced a 51% increase in total cholesterol level in the liver, a small but significant increase in intestinal cholesterol, and no change of this level in kidney. At the same time there was a striking decrease in the rate of hepatic cholesterol synthesis. Despite

TABLE I. Effect of Bile Acids on Cholesterol Levels in Mouse Tissues.

	Total cholesterol, mg/g* tissue		
Group	Liver	Small intestine	Kidney
A. Control	5.14 ±1.10	2.21 ±.20	4.42 ±.39
B. Cholic acid, .5%	7.76 ±1.74	2.80 ±.18	4.58 ±.42
C. Hyodeoxycholic acid, .5%	2.82 ± .38	2.23 ±.16	4.43 ±.31
D. Deoxycholic acid, .5%	3.88 ±1.16	1.49 ±.54	3.96 ±.28
E. Lithocholic acid, .5%	3.28 ± .75		

Statistical comparison of values:

Groups	Liver	Intestine	Kidney
A and B	$P < .01$	$P < .01$	$P = .38$
A " C	"	$P = .80$	$P = 1.00$
A " D	$P = .03$	$P < .01$	$P < .01$
A " E	$P < .01$		

* Wet wt.

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* This investigation was supported, in part, by grant from Michigan Heart Assn.

TABLE II. Effect of Bile Acids on Incorporation of Acetate-1-C^{14} into Mouse Tissue Cholesterol.

	Activity of cholesterol from 1 g* tissue, counts/min.	
Group	Liver	Small intestine
A. Control	1,806 ± 508	3,055 ± 587
B. Cholic acid, .5%	900 ± 172	3,244 ± 614
C. Hyodeoxycholic acid, .5%	7,283 ±1,532	5,484 ±1,104
D. Deoxycholic acid, .5%	230 ± 75	
E. Lithocholic acid, .5%	4,065 ±1,700	

Statistical comparison of values:

Groups	Liver	Intestine
A and B	$P < .01$	$P = .52$
A " C	"	$P < .01$
A " D	"	
A " E	"	

* Wet wt.

the small increase of intestinal cholesterol, incorporation of acetate-1-C^{14} into cholesterol was unchanged.

Hyodeoxycholic and lithocholic acids effected significant decreases in hepatic cholesterol levels (45 and 36%), while greatly increasing incorporation of acetate-1-C^{14} into hepatic cholesterol. Hyodeoxycholic acid did not alter levels of intestinal or kidney cholesterol, but appeared to increase the synthesis rate of cholesterol in the small intestine of animals sacrificed one hour after injection. However, this increase in cholesterol-x-C^{14} concentration over control level does not represent a real increase in intestinal synthesis rate, since the cholesterol-x-C^{14} level of the treated animals as compared with the controls was not significantly increased in groups sacrificed 15 minutes after injection (Table III). Thus, the increased cholesterol-x-C^{14} level at the one hour interval originated extra-intestinally. A similar consideration makes it apparent that observed increases in cholesterol-x-C^{14} levels in extra-hepatic tissues of mice sacrificed an hour after injection do not indicate real increases in synthesis rates. On the other hand, in the case of liver, the data at 15 minutes and 1 hour show that there is a real increase in synthesis rate in this organ.

Deoxycholic acid differed from the other bile acids inasmuch as it produced significant decreases in liver, intestine and kidney cholesterol levels, along with a large decrease in hepatic cholesterol synthesis rate. These directly proportional effects on cholesterol levels and synthesis rates indicate that this bile acid does not act by the same mechanism as the other bile acids, and should be further investigated.

These experiments, together with previous investigations(1,2,3), have determined the general effects of 5 dietary bile acids on cholesterol metabolism in mouse or rat. With the exception of deoxycholic acid, the effect of each of these substances on the incorporation rate of acetate-1-C^{14} into liver cholesterol is inversely related to the changes in hepatic cholesterol concentration. These findings suggest that, as in the case of cholic acid(3), the effects of various bile acids on cholesterol synthesis reflect their effects on hepatic cholesterol levels. Inasmuch as some of these substances are representative of the major bile acids produced during cholesterol catabolism, their role in homeostatic control of liver cholesterol metabolism can be considered significant. It must be emphasized that however effective these substances are in altering hepatic cholesterol metabolism, all other tissues investigated, except adrenal(1), were not altered to any great extent. This is especially interesting in the cases of cholic and

TABLE III. Cholesterol-x-C^{14} Levels in Liver and Intestine 15 Minutes and 1 Hour after Injection of Sodium Acetate-1-C^{14}.

	Activity of cholesterol from 1 g* tissue, counts/min.			
	Liver		Small intestine	
Group	15 min.	1 hr	15 min.	1 hr
F. Control	2,629 ± 697	1,806 ± 508	3,837 ± 1,196	3,055 ± 587
G. Hyodeoxycholic acid, .5%	6,325 ± 1,040	7,283 ± 1,532	4,412 ± 895	5,484 ± 1,104
Statistical comparison of values:				
Groups F and G	$P < .01$	$P < .01$	$P = .25$	$P < .01$

* Wet wt.

hyodeoxycholic acids, which bring about striking changes in hepatic cholesterol levels and synthesis rates in the mouse, but have little or no effect on small intestine or kidney. It would appear that in the mouse or rat, homeostatic control of cholesterol metabolism in kidney and intestine is more or less independent of hepatic control and of the bile acids influencing cholesterol synthesis in the liver.

Summary. Feeding various bile acids to mice for 3 weeks had the following effects: 1. Cholic acid increased hepatic and intestinal cholesterol levels, but had no effect on kidney cholesterol. It decreased hepatic cholesterol synthesis rates, but had no effect on intestinal synthesis rates. 2. Hyodeoxycholic and lithocholic acids decreased liver cholesterol levels. Hyodeoxycholic acid had no effect on intestine and kidney cholesterol, or on intestinal cholesterol-x-C^{14}. Both acids effected large increases in hepatic cholesterol synthesis. 3. Deoxycholic acid significantly decreased liver, small intestine and kidney cholesterol levels. It also decreased hepatic cholesterol synthesis. 4. Results of this study suggest that homeostatic control of cholesterol metabolism in tissues other than liver is independent of hepatic control.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Relative Abundance of Vasopressin and Corticotrophin-Releasing Factor in Neurohypophysial Extracts.* (25233)

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In our previous experiments using rats with median eminence lesions to assay adrenocorticotrophin (ACTH)-releasing activity, the ACTH-releasing potency of posterior pituitary preparations was accounted for by their vasopressin content(1,2). Saffran and Schally (3) and later Guillemin and co-workers(4) reported, on the other hand, that extracts of posterior lobe of the pituitary gland contain a corticotrophin-releasing factor (CRF), distinct from vasopressin, which releases ACTH from pituitaries, *in vitro*. Attempts to demonstrate activity of this CRF of Guillemin *in vivo* were unsuccessful(1) using rats with hypothalamic lesions. Recently, Royce and Sayers(5) reported the presence of a CRF distinct from vasopressin in extracts of calf stalk-median eminence tissue, assaying the activity in rats with median eminence lesions. This observation prompted a reinvestigation of the ACTH-releasing activity of various brain extracts from hypothalamus and other regions, utilizing rats with lesions to assess ACTH-releasing activity.

Methods. Preparation of animals. Hypothalamic lesions were made in the median eminence of the tuber cinereum using methods previously reported(1) except that the rats were injected intramuscularly with 60,000 U crystalline procaine penicillin G at operation. Rats drinking at least 100 ml of water during the first 24 hours post-operatively were selected for the assay procedure. This assay of ACTH-releasing activity was performed 48 hours after placing the lesions, since at that time such rats have been shown to be unresponsive to a variety of non-specific stimuli (1-2). Proper selection of coordinates can

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* Supported in part by Public Health Service Grants to S. M. McCann and Dr. Lewis Flexner.

give nearly 100% successful operations using the above criteria. These animals are called rats with "acute" lesions. *Preparation of brain extracts.* Beef cortex and stalk-median eminence tissue were obtained at the slaughter house and placed in a beaker on dry ice. On arrival in the laboratory, the tissue from 20 brains was weighed, 40 ml of 0.2 M acetic acid were added, and the mixture was homogenized in a Waring blendor for 120 seconds. The mixture was refluxed for 10 minutes in a 100°C water bath and then centrifuged at 16,000 rpm for one hour in a Spinco ultracentrifuge(5). The supernatant was injected into assay animals. Rat stalk-median eminence tissue or brain stem was obtained from hypophysectomized rats, 24-48 hours after hypophysectomy, and ground in 0.5 ml 0.1 N HCl. One-tenth N HCl was added to pools of 6-12 stalk-median eminences to bring the volume to 0.5 ml per stalk-median eminence. After thorough mixing, the mixture was centrifuged at 3,000 rpm for 15 minutes, and the supernatant was injected into assay rats. *Bioassay for ACTH, ACTH-releasing, and pressor activity.* i) Assay for ACTH concentration of the extracts was performed in hypophysectomized rats,† 18-24 hours post-hypophysectomy, by the method of Sayers *et al.* (6) using USP reference standard corticotrophin as the standard. ii) ACTH-releasing activity was assessed in rats with "acute" lesions using adrenal ascorbic acid depletion as the index of ACTH activity(1). The left adrenal was removed under ether anesthesia to obtain a control value for adrenal ascorbic acid. One hour after intravenous injection of a test drug, the second gland was removed for analysis. iii.) Pressor activity of the extracts was measured in the urethane-anesthetized, dibenamine-treated rat as described by Dekanski (7). Statistical analysis was performed by standard methods for bioassays(8).

Results. Comparison of sensitivity to ACTH of hypophysectomized rats and those with "acute" lesions. Since ACTH content of the extracts of hypothalamic tissue was assessed in hypophysectomized rats, whereas ACTH-releasing activity was evaluated in rats with lesions, it was important to compare the sensitivity of these two types of rats to ACTH. The results of a multidose assay (Fig. 1) indicate that rats with lesions are 3 times less sensitive to ACTH than the hypophysectomized animals. Rats with lesions were of the Wistar strain while the hypophysectomized rats were Sprague-Dawleys. Consequently, the experiment was repeated using Sprague-Dawley rats with lesions to determine if a strain difference in sensitivity to ACTH was responsible for the results. Eight Sprague-Dawley rats with lesions also showed a diminished sensitivity to ACTH when tested at 2 dose levels. The difference in sensitivity to ACTH between hypophysectomized rats and those with lesions must be taken into consideration in evaluating the contribution made by ACTH to response to hypothalamic extracts in rats with lesions.

Activity of vasopressin in rats with "acute" lesions. To determine the contribution of vasopressin to activity of hypothalamic extracts, it was necessary to measure the ascorbic acid depletion resulting from injection of vasopressin in rats with "acute" lesions. The results obtained previously(2) with Pitressin and synthetic lysine-vasopressin are shown in Fig. 2. Responses to the 2 substances were similar when dose was expressed in pressor units. The ascorbic acid depletion resulting from administration of 0.1 U or 0.5 U of Pitressin (4 rats at each dose) in the present experiments did not differ significantly from that obtained previously. Consequently, these earlier results with lysine-vasopressin have been used to evaluate the contribution of vasopressin to the activity of the hypothalamic extracts. Royce and Sayers (9) reported that part of the activity of Pitressin in rats with "acute" lesions was due to an extra-pituitary action of vasopressin. This conclusion was based on their finding that Pitressin evoked ascorbic acid depletion in rats with "acute" lesions, which had been decapitated just prior to injection of the drug. Since our animals with lesions appear to be considerably less sensitive to vasopressin than theirs, it appeared of interest to test the effect of Pitressin in decapitated rats with "acute" lesions, as they had done, to determine if the ascorbic acid depletion induced by Pitressin could be accounted for by its effect in the decapitated rat having no pituitary gland. A dose of 0.5 U Pitressin, which induced an adrenal ascorbic acid depletion of 86 ± 13 mg % in 10 rats with "acute" lesions, produced a fall of only 30 ± 14 mg % in the ascorbic acid of 9 decapitated rats. The response of the decapitated rats was not significantly different from zero ($P<0.1$) and is significantly less than that found in rats with lesions ($P<0.01$). (The difference in response of the 2 types of rat is about the same as that reported by Royce and Sayers.) Failure to induce a significant depletion with Pitressin in the decapitated rat may have been related to the relatively low dose of Pitressin employed.

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† Obtained from Hormone Assay Laboratories, Chicago, Ill.

ACTH-releasing activity of beef stalk-median eminence extract (S.M.E.) This extract proved active in both the rat with lesions and the hypophysectomized rat (Table I). The minimal effective dose of 0.0032 of a single stalk-median eminence was derived from 0.5 mg wet weight of tissue. Ascorbic acid depletions obtained in the 2 types of assay animal were indistinguishable; however, since the rats with lesions were much less sensitive to ACTH than the hypophysectomized rats, statistical treatment of the data indicates that the ACTH content of the extract was 290 (167-502)‡ mU of ACTH/SME as assayed in the hypophysectomized rat, whereas the apparent ACTH potency in rats with lesions was 733(440-1200)‡ mU ACTH/SME (Table III). Thus, ACTH contamination of the extract accounted for only 40% of its activity in rats with lesions and a significant, additional, ACTH-releasing activity was demonstrated.

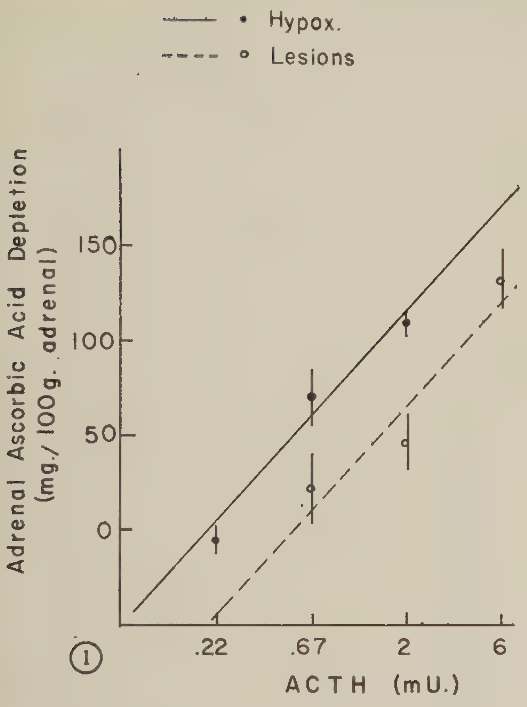

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FIG. 1. Comparison of sensitivity to ACTH of hypophysectomized rats and those with lesions. Vertical lines indicate stand. errors. Each point is the mean of 6 or 7 determinations.

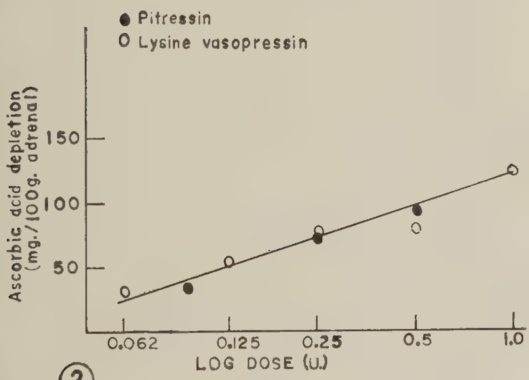

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FIG. 2. Comparison of activity of Pitressin and synthetic lysine-vasopressin in rats with lesions. Dosage is in pressor units. Reproduced from paper of McCann and Fruit(2).

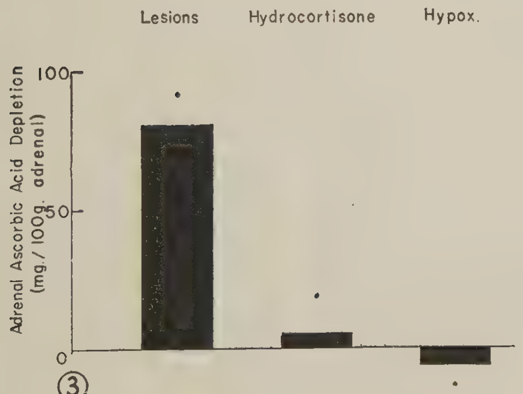

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FIG. 3. Adrenal ascorbic acid depletion induced by purified CRF of Royce and Sayers. Hypox. refers to hypophysectomized rats. Hydrocortisone refers to animals inj. with hydrocortisone. Dots above the bars indicate stand. errors.

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‡ 95% confidence limits of the assay.

TABLE I. ACTH-Releasing Activity of Extracts of Beef Brain.

				Adrenal ascorbic acid depletion, mean ± SEM (mg/100 g)	
Extract	Dose, U*	Wet wt of tissue, mg	Pressor activity, mU	Median eminence lesions	Hypophysectomized
Stalk-median eminence	.0125	2		168 ± 16 (5)‡	157 ± 17 (6)
	.0032	.5	13 (11–16)†	78 ± 27 (6)	66 ± 29 (5)
	.0016	.25		16 ± 19 (5)	
Cortex	1.0	200		111 ± 8 (4)	
	.1	20		14 ± 10 (5)	
	.0125	2		6 ± 14 (4)	

* 1 U is one stalk-median eminence or equivalent weight of cortex.
† 95% confidence limits.
‡ No. in parentheses is No. of rats in group.

The vasopressin content of 13(11-16)‡ mU at the minimal effective dose of extract could hardly have been responsible for this ACTH-releasing activity for the following reasons. Assuming that the pressor assay gives a valid estimate of the vasopressin content of the extracts, comparison of the responses to synthetic vasopressin and to beef extract in rats with lesions leads to the conclusion that only 5(2-10)‡ % of total activity of the extract can be due to vasopressin (Table III). Adding this 5% to the 40% accounted for by presence of ACTH leaves 55% of the activity presumably due to a CRF distinct from vasopressin.

An attempt was made to purify the active principle by selectively adsorbing the ACTH and vasopressin from this extract (and from a similar one obtained from calf tissue) with oxycellulose(5). Unfortunately, in our hands the oxycellulose almost quantitatively adsorbed all active material.

To determine the specificity of the responses to SME, an extract of cerebral cortex was prepared similarly and assayed in rats with lesions. The dose in units given in Table I represents a similar weight of tissue to that used to prepare SME. The dry weight of the extract was also comparable. No response was obtained until the dose was increased approximately 200 fold over that required for SME (Table I).

ACTH-releasing activity of stalk-median eminence extracts from hypophysectomized rats. SME from hypophysectomized rats was prepared and assayed in order to test extracts from another species and to eliminate ACTH contamination from the extracts. The results (Table II) indicate that one SME induced a significant ascorbic acid depletion in rats with lesions. Two SME's gave a marked depletion when no response was elicited in the hypophysectomized rat. Thirty-four mU of vasopressin were found per SME, indicating that 21(8-52)‡ % of the activity of this extract could be due to vasopressin. The remaining 79% was presumably accounted for by CRF (Table III). Extract prepared from an amount of the brain stem of hypophysectomized rats equivalent to the larger dose of stalk-median eminence was inactive in rats with lesions.

Corticotrophin-Releasing Factor of Royce and Sayers. An extract of calf stalk-median eminence purified by Royce and Sayers and

TABLE II. ACTH-Releasing Activity of Extracts of Rat Brain.

			Adrenal ascorbic acid depletion, mean ± SEM (mg/100 g)	
Extract	Dose, U*	Pressor activity, mU	Median eminence lesions	Hypophysectomized
Stalk-median eminence	1	34 (26–43)†	40 ± 13 (9)‡	
	2		110 ± 5 (6)	14 ± 6 (6)
Brain stem	2		8 ± 11 (5)	

* 1 U is one stalk-median eminence.
† 95% confidence limits.
‡ No. in parentheses is No. of rats in group.

TABLE III. Summary of ACTH-Releasing Activity of Stalk-Median Eminence Extracts.

Extract	ACTH content (hypox. rats)	ACTH-like activity (lesion rats)	Vasopressin content	Estimate of % ACTH-like activity due to:		
	mU/SME			ACTH	CRF	Vasopressin
Beef	290 (167–502)[*]	733 (440–1200)[*]	4150 (3500–5000)[*]	40	55	5 (2–10)[*]
Rat, hypox.	≦ 0.2	3	34 (26–43)[*]	0	79	21 (8–52)[*]
Purified CRF (Royce and Sayers)	< ”	7	37 (30–40)[*]	0	~96	~4

[*] 95% confidence limits.

kindly provided to us proved active in rats with lesions at a dose of 0.3 SME (Fig. 3). It was inactive in the hypophysectomized rat and contained only 11(9-12)[‡] mU of vasopressin per 0.3 SME. Consequently, approximately 96% of the activity of this extract was attributable to CRF (Table III). This extract was inactive in the hydrocortisone-inhibited rat(10), but this absence of effect may have been due to the decreased sensitivity to ACTH of the steroid blocked rat since these rats gave a response of only 82 ± 10 to 6 mU of ACTH. This latter response is significantly less than that given by rats with lesions (133 ± 14; $P<0.02$).

Stability of ACTH-releasing activity. The activity of beef extract appeared to be quite stable in acid solution since refluxing for 10 minutes did not abolish it (see Methods), and since the extract was stable on storage at 4°C for 4 months. A dose of 0.0125 SME which initially gave an ascorbic acid depletion of 168 ± 16 (Table I) gave a response of 147 ± 7 when retested 4 months later in 5 rats. Extracts from rat hypothalamus likewise appeared to be stable at 4°C.

Location of lesions. In view of our wide experience with this particular lesion, study of the brains of these animals was restricted to gross examination, which indicated that the lesions were located in the basal hypothalamus in the region between optic chiasm and pituitary stalk. Serial sections of pituitaries from 8 rats revealed tissue which appeared normal and no infarcts were seen.

Discussion. In the present study rats with lesions in the median eminence were less sensitive to ACTH when tested 48 hours post-operatively than hypophysectomized rats tested 24 hours after operation. This is in contrast to the findings of Royce and Sayers that rats with lesions were at least as sensitive to ACTH as the hypophysectomized animal when tested under conditions apparently similar to those employed here(5). The reason for this difference is not apparent. The hypophysectomized rat is known to lose its sensitivity to ACTH under the conditions of the Sayers' assay with the passage of time, presumably because of absence of ACTH secretion(11). Similarly, the decreased output of ACTH in rats with lesions might have made these rats less sensitive than the hypophysectomized rats which were tested after a shorter post-operative interval. In a previous study from this laboratory(1), rats tested 48 hours after either lesions or hypophysectomy gave equivalent responses to a rather high dose of 5 mU of ACTH. This result is at least consistent with the suggestion that the decrease in ACTH sensitivity of rats with lesions in comparison with hypophysectomized rats tested in the present experiments may have been due to the greater time which had elapsed after operation in the rats with lesions. Other unknown causes may also have contributed to the diminished sensitivity of animals with lesions.

Since the rat with lesions is less sensitive to ACTH than the hypophysectomized rat, the question arises as to the role of decreased ACTH sensitivity in producing the block of ascorbic acid depletion in response to stress found in rats with lesions. Though decreased sensitivity to ACTH may be a factor in producing this failure of ascorbic acid depletion, the ascorbic acid depletion induced in normal rats by a stress such as unilateral adrenalectomy is of such a magnitude(12) as to suggest discharge of sufficient ACTH to deplete

TABLE IV. ACTH-Releasing Potency of Various Neurohypophysial Extracts.

Extract	% ACTH-releasing activity due to Vasopressin	CRF
Beef SME	9 (4– 18)*	91
Rat "	21 (8– 52)*	79
Protopituitrin†	141 (42–472)*	~ 0
Pitressin†	121 (46–319)*	~ 0
Purified CRF of Royce & Sayers	~4	~96

* 95% confidence limits.
† Calculated from data of McCann(1,2).

ascorbic acid even in the less sensitive rat with lesions. Failure of ascorbic acid depletion in rats with lesions would then be due both to reduced secretion of ACTH by such rats(13, 14) and to reduced sensitivity of the adrenal to the ACTH released.

The results presented here indicate that crude extracts from the stalk-median eminence region of beef and rat brain produce in rats with hypothalamic lesions a significant release of ACTH which cannot be accounted for by their content of vasopressin, thus, confirming the findings of Royce and Sayers(5). That this is a specific response to stalk-median eminence tissue is indicated by the lack of activity of extracts from beef cortex and rat brain stem when injected in comparable amounts. (The effect of beef cortex at a dose 200 times greater than that required with SME cannot be explained at this time. It may be a non-specific effect; on the other hand the lack of apparent toxicity of even this large dose of extract argues against this interpretation. A possible explanation is contamination of the extract with ACTH or CRF.)

The relative contribution of vasopressin and CRF to the ACTH-releasing activity of various neurohypophysial extracts is given in Table IV. No correction has been made for the relatively small proportion of the effect of vasopressin which can be accounted for by its action at extrahypophysial sites(9,15). It would appear that vasopressin can account for only 10-20% of the ACTH-releasing activity of the stalk-median eminence area, whereas it accounts for all of the activity found in posterior lobe extracts within the limits of error of the methods. The CRF found in posterior lobe extracts by Saffran and Schally(3) and by Guillemin and coworkers(4) would seem to be present in relatively small amounts. There appears then to be a differential localization of CRF and vasopressin in these 2 portions of the neurohypophysis.

Since lesions in the median eminence block ACTH release, whereas ACTH release can be induced in such animals by extracts from this same region containing vasopressin and CRF, it is concluded that vasopressin and CRF share the role of ACTH-releasing neurohumor. If storage in tissue is any criterion of secretion rates, it would appear that CRF is the principal ACTH-releasing substance from stalk-median eminence but that vasopressin is the chief factor in neural lobe. Secretion into hypophysial portal vessels is probably the critical factor in providing a sufficient concentration of neurohumor to the pars distalis. From this standpoint, CRF appears to be located in a more strategic position at site of origin of these vessels in the median eminence and stalk. However, vasopressin might reach the pars distalis in high concentration *via* the short portal vessels which arise in the neural lobe and drain blood to the pars distalis(16). Only further studies of the ACTH-releasing activity of portal vessel blood from these 2 sites and more information concerning the proportion of hypophysial blood flow coming from these 2 types of portal vessels can conclusively establish the relative importance of CRF and vasopressin for control of ACTH secretion.

Conclusions. Extracts of the stalk-median eminence area of beef or rat brain produce a specific release of ACTH in rats with hypothalamic lesions. The major portion of this effect is due to a corticotrophin-releasing factor. Ten to 20% of the activity of these extracts appears to be due to vasopressin.

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Received July 8, 1959. P.S.E.B.M., 1959, v102.

Effect of Temperature Extremes and Cortisone on Toxicity of Diphosphopyridine Nucleotide in Mice. (25234)

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It was reported recently(1) that analogs of nicotinamide cause toxic effects in mammals. Degenerative changes in the central nervous system appeared as prominent features of intoxication in mice and rats. Selective neuropathological damage was seen in nuclei of the brain stem and in gray matter of the anterior horn. Kaplan and coworkers(2) showed that analogs of nicotinamide participate in enzymatically catalysed exchange reactions with diphosphopyridine nucleotide (DPN), and they suggested that the toxicity of analogs is due to such reactions. These findings were confirmed and extended by Burton(3). Burton and others(4) could also demonstrate that reserpine and promazine prolong elevation of DPN concentration of tissues caused by injection of nicotinamide in mice. To examine DPN effects in a different manner, we determined first the toxicity of injected DPN. Then we investigated the effect of injected DPN on mice, as animals were subjected to stress of extreme environmental temperatures. Glock and McLean(5) found that starvation or administration of a variety of hormones alter DPN content of tissues considerably. It is well established that under stress of temperature extremes, especially cold, activity of the adrenal cortex increases significantly. Scherr reported(6) that toxicity of cortisone in mice varies according to environmental temperatures. After repeating his experiments we administered DPN and cortisone simultaneously, for evidence was offered by Munch *et al.*(7), that there is a specific interaction between steroid hormones and coenzymes like DPN.

Method. DPN was injected intraperitoneally to 20 Swiss mice, each weighing 24 g. The animals were observed behaviorally and histological studies of brain stem, muscles, kidneys and liver were performed.

Result. 105 mg of DPN/animal killed all mice in 4 hours. Before death, animals exhibit dysmetria, paralysis of hind legs, and marked general irritability. Neuropathological findings were similar to those described by Sternberg and Philips(1). Striped muscles, kidneys or liver showed no histopathological changes. Fifty mg of DPN is well tolerated intraperitoneally. Daily injection of this dose for 15 days, or more, produced no deleterious effects.

DPN injection and environmental temperatures. Method. Swiss mice, each weighing 24 g, in groups of 10, were exposed to hot environmental temperatures (37°C). Others, also in groups of 10, were subjected to cold environmental temperatures (7°C). They received 50 mg of DPN intraperitoneally. Control groups did not receive DPN.

Result. Mice exposed to environmental

temperature of 37°C, others to 7°C, in groups of 10, endured them without mortality. 50 mg of DPN intraperitoneally caused no mortality at room temperature. 50 mg of DPN became acutely toxic for mice at extreme temperatures. At 37°C it caused 50% mortality in 24 hours and 100% mortality in 72 hours. At 7°C it caused 50% mortality in 4 hours and 100% mortality in 24 hours.

DPN injection and cortical steroid. Method. Mice, in groups of 10, were given 0.5 mg cortisone intraperitoneally at room temperature and at temperature extremes. Other groups were administered 50 mg of DPN and 0.5 mg of cortisone simultaneously at room temperature and at temperature extremes.

Result. 0.5 mg of cortisone is well tolerated at room temperature. The same amount became lethal at high temperature. This confirms the results of Scherr(6). At 37°C it caused 50% mortality in 48 hours and 100% mortality in 144 hours. Toxicity of DPN is significantly altered by simultaneous cortisone administration. At room temperature 50 mg of DPN with simultaneous cortisone, each of which alone causes no mortality, produces marked lethal effects. The combined effect of DPN and cortisone is most pronounced under stress of cold environment. At 37°C they produced 50% mortality in 4 hours and 100% mortality in 38 hours. At 7°C they caused 50% mortality in 2 hours and 100% mortality in 6 hours. At room temperature they caused 50% mortality in 6 hours and 100% in 48 hours.

Discussion. We showed that DPN given intraperitoneally in mice has marked toxic effects. DPN proved especially toxic when animals were subjected to stress of hot or cold environment. These toxic effects are much enhanced by simultaneous administration of cortisone. Our data are in good accord with the view(7) that steroid hormones and coenzymes form a steroidcoenzyme complex.

McIlwain and Rodnight(8) showed that brain tissue DPN-ase activity is exceptionally high. They suggested that in unimpaired brain tissue the rate of DPN decomposition is checked by special inhibitors and by peculiarities of cellular architecture. Burton and others reported(4) that brain DPN content increases with nicotinamide injection, and this increased level is further extended by administration of reserpine or promazine. They also found that compounds related to reserpine or promazine which are not "tranquilizing" agents, fail to extend the elevated DPN levels. Thus tranquilizing effects seem to inhibit DPN-ase activity of tissues. Furthermore, DPN requiring phosphorylations are also inhibited(9) by tranquilizing compounds. Thus it is likely that tranquilizing agents act selectively in such manner that particular brain tissue DPN-ase is inhibited. A recent report of Woolley(10) comes to similar conclusion. He studied tranquilizing and antiserotonin activity of nicotinamide. He found evidence that inhibition of DPN-ase is a likely mechanism of the antiserotonin and tranquilizing action of large doses of nicotinamide. Stress itself demands an increase in DPN requiring phosphorylations(11,12).

Civen and Knox reported(13) that tryptophan and/or hydrocortisone treatment produced a marked increase in tryptophan-transaminase levels of the liver. This "stress of substrate injection"(13) was potentiated by injection of hydrocortisone. In an analogous manner, our findings, support the hypothesis that stress is concomitant with increase of DPN-ase activity and reduction of DPN-ase activity parallels reduction of stress.

Summary. 1. DPN injection produces toxic effects in mice similar to those described for nicotinamide analogs. 2. Toxicity of injected DPN is influenced by environmental temperatures; both cold and hot environment enhances toxicity. 3. Simultaneous cortisone potentiates the effect of heat and cold. 4. These effects are discussed as they bear on inhibition of DPN-ase activity of reserpine, promazine and nicotinamide.

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Received July 10, 1959. P.S.E.B.M., 1959, v102.

Effect of Desiccated Thyroid and Thiouracil on Dental Caries Experience of Adult Rats.* (25235)

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Many previous studies(1-7) from these laboratories have shown that feeding desiccated thyroid to rats reduces the incidence of dental caries and administration of either I^{131} or propylthiouracil increases the caries experience. In all previous studies, the thyroid drugs were administered to weanling rats and continued for periods varying from 90 to 140 days. Since previous experience has shown that the proteolytic activity of the secretions of salivary glands of control animals increases with age, but that the effect of propylthiouracil on proteolytic activity is independent of age(8), it was considered of importance to study the relationship between thyroid activity and dental caries in adult animals. Since the proteolytic activity of the salivary glands increases with age with continued administration of desiccated thyroid(8), such information may be helpful in explaining the mechanism of action of the thyroid gland on the caries experience of the rat.

Method. A total of 120 weanling male Sprague-Dawley strain rats was divided equally into 3 groups according to body weight. All of the animals were housed in pairs in raised screen cages and placed on a low caries producing diet (this diet would produce about a mean of 1.5 carious lesions in 100 days) consisting of (in g %): granulated sugar, 72; vitamin test casein, 18; cottonseed oil, 5; vitamin mixture, 1; salts, 4; plus 20 drops of Percomorph oil per kilo of diet. After 100 days (123 days of age) they were divided into 3 groups: Group I animals received a stock corn cariogenic diet consisting of (in g %): yellow corn grits, 52.7; ground yellow whole corn, 11.3; powdered whole milk, 30; alfalfa, 4.8; iodized sodium chloride, 1; and, irradiated yeast, 0.2. The animals in Group II received the same diet to which 0.1% proplythiouracil had been added, and those in Group III the same diet as those in Group I except that it contained 250 mg of desiccated thyroid per kilo of diet. All of the animals received fluoride-low (F = 0.05 mg/ml) drinking water *ad libitum* and remained on their respective diets for an additional 100 days. They were then sacrificed, their heads prepared for dental caries evaluation by methods previously described(9), and thyroid and salivary glands removed for histologic examination.

Results. The data pertaining to body weight gain of the animals and the effect of thyroid drugs on the caries experience are seen in Table I. As previously reported, administration of propylthiouracil is associated with a failure to gain weight. In this study the final body weight of the control group had a mean value of 333 g while that of the animals receiving thiouracil was 214 g. In previous studies the animals receiving desiccated thyroid similarly failed to gain as much weight as the controls, due undoubtedly to their high rate of metabolism. In this study, however, amount of desiccated thyroid was adjusted so that their weight gain was comparable to the controls.

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* This study supported in part by grant from U.S.P.H.S.

TABLE I. Effect of Desiccated Thyroid or Propylthiouracil on Body Weight and Dental Caries Experience of Adult Rats.

			Dental caries experience			
Group	No. of rats	Final body wt (g)	Mean No. of lesions	p*	% rats with caries	Mean severity index of lesions
I (control)	16	333	2.4 ± .09†		81	2.00
II (thiouracil)	28	214	5.5 ± .08	.01	95	2.41
III (thyroid)	28	330	2.4 ± .05		60	1.71

* Calculated on basis of T-test. † Stand. dev.

The incidence of dental caries is also shown in Table I. Controls had a mean caries experience of 2.4 lesions, those receiving propylthiouracil 5.5, and those which received the desiccated thyroid 2.4. The group receiving thiouracil is significantly ($p = 0.01$) different from the controls. The lesions of animals receiving thiouracil were the largest, and those receiving the desiccated thyroid were the smallest.

Discussion. The control animals had an extremely low incidence of dental caries even though they received a highly cariogenic diet for 100 days. This finding is in keeping with our previous findings(10) that rats must be placed on the cariogenic diet before 30 days of age in order to produce a high caries incidence. More attention should be given to those factors which affect the physical or chemical nature of the erupted tooth making the dentition less susceptible to experimentally produced caries as the animals increase in age. It may also be important to consider what environmental factors are changed as well as the changes in the salivary tissues as the animals increase in age, since it has been shown that both salivary flow, viscosity, and pH are a function of the age of the animal(11).

The cariogenic effect of propylthiouracil, as demonstrated so repeatedly in weanling animals, is evident even in adult rats. The mechanism responsible for modifying salivary gland function is, therefore, still operable in adults, thus suggesting that the effect of the propylthiouracil on salivary tissue is not dependent on age. Not only is propylthiouracil associated with an increase in mean number of lesions, but its cariogenic effect is shown also by a higher number of animals with caries as well as by a higher severity index of the lesions.

The anticariogenic effect of desiccated thyroid is not present in the adult rats. This may be due to the fact that such a low dosage level of desiccated thyroid was required not to offset body weight gain of the animals. In earlier studies much higher levels were used. Furthermore, the most dramatic anticariogenic effect of administering desiccated thyroid or thyroxin results when these drugs are given to animals whose thyroid activity had been previously diminished by either I^{131} or propylthiouracil. This certainly is in keeping with the hypothesis that administering thyroxin or desiccated thyroid to an animal with "normal" thyroid function should not result in such dramatic dental changes as would be found in an animal with hypothyroidism. However, even at the low dosage levels of desiccated thyroid used in this study, one can see evidence of the anticariogenic effect of the thyroid drug. For example, the number of rats with caries is much less as is the severity of the lesion.

Histologic examination of the thyroid glands of animals receiving propylthiouracil revealed the typical extreme hyperplasia of the gland with increased cellularity and lack of colloid formation. In contrast, the animals receiving thyroid exhibited small thyroid glands with follicles lined by flattened cells and filled with colloid.

The salivary glands of animals in this study did not differ from those in previous studies on younger animals. The granular tubules of the submaxillary glands in animals receiving propylthiouracil were extremely small and nearly devoid of granules while those receiving thyroid were extremely large and choked with granules. No changes were noted in either the parotid or sublingual glands of either group.

From histologic evidence alone, there does not appear to be significant difference in reaction of either the thyroid gland or the submaxillary gland to desiccated thyroid or to propylthiouracil between young weanling or older adult animals.

Conclusions. The effect of feeding either desiccated thyroid or propylthiouracil was studied to determine if the former drug resulted in an anticariogenic effect and the latter produced a cariogenic effect in adult rats to the same degree as in weanling animals. Propylthiouracil resulted in significantly more caries than were found in weanling animals, but desiccated thyroid did not reduce the caries experience. Similar histologic changes were found in both thyroid and salivary glands in the adult rats as were found previously in weanlings.

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Received July 10, 1959. P.S.E.B.M., 1959, v102.

Effects of High Pressures on Influenza Viruses.* (25236)

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Various viruses have been subjected to high compression pressures of 30,000 to 120,000 lb/in^2 (2000 to 8000 atmospheres) to test the effect of these pressures on infectivity, but the results were complicated by temperature increases accompanying the pressure rise. Since pressure was generated by a hydraulic press operated at room temperature, increase in heat of the virus suspension in the press was unavoidable. Using the hydraulic press apparatus Basset(1,2) studied a number of viruses including 2 plant agents (tobacco mosaic and tobacco mecrosis), 6 animal (vaccinia, herpes, rabies, yellow fever, Rous sarcoma, and equine encephalomyelitis) and staphylococcus bacteriophage. The most susceptible to inactivation was the bacteriophage which was inactivated at 30,000 lb/in^2 and the most resistant was tobacco mosaic inactivated at 12,000 lb/in^2. All of the above studies were reported prior to 1941 so that much modern data regarding measurements of infectivity were not available. An ingenious device recently developed by Arthur D. Little produces pressures of over 30,000 lb/in^2 at below freezing temperatures. The method depends on freezing and expansion of water in a steel cell. The sample to be tested is placed inside the cylinder which is then filled with water. The cell is sealed and placed in a deep freeze at various freezing temperatures. As the water freezes it expands against the contracting steel and internal compression pressures result which can be measured by a strain gauge. Using this device Curtis *et al.*(3) studied avian erythroblastosis and mouse hepatitis (Nelson) viruses and found inactivation complete at 30,000 and 25,000 lb/in^2 respectively. Experiments to determine minimum pressure were not reported. None of the previous studies of the effects of pressure on viruses have included influenza virus. Since this virus is represented by many diverse strains all of which have 2 distinct and easily

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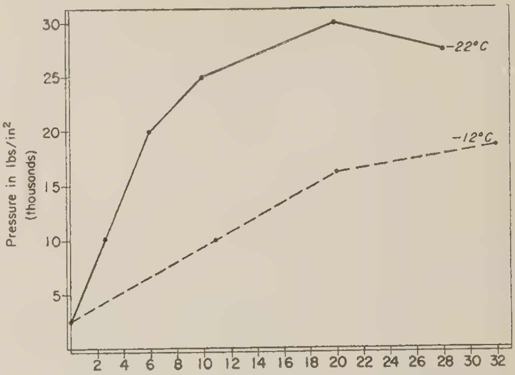
* This work supported in part by Grant from U.S.P.H.S.

measured biological properties, hemagglutinin and infectivity, it was of interest to investigate the effects of pressure developed at below freezing temperatures on these agents.

Materials and methods. Viruses. Virus strains used included SW, PR8, FM-1, Lee, IB BCal, and 1233 ("C") all obtained from the Army Medical School, Walter Reed Medical Center, Washington, D. C. The 1233 "C" strain was maintained by amniotic passage; all others were allantoic adopted strains. *Hemagglutinin and infectivity.* Hemagglutinin titrations were performed in the usual manner at temperature indicated using 0.5% chicken red blood cell suspension. Infectivity titrations were made by inoculation of full-log dilutions into groups of 4-6 eggs. The eggs were then tested for the presence of hemagglutinin and endpoints calculated by the Reed and Muench method. *Pressure studies.* Virus suspensions to be subjected to pressure were placed in a soft plastic tube 3-4″ long and sealed so that no air bubbles were present. Water to be used in filling the cell was first boiled to remove all possible absorbed air and then chilled to 7°C. Likewise the steel cell itself was chilled to 7° prior to use. Virus samples were then placed in the chamber and this filled with water which was then overlaid with hydraulic oil as a seal. The steel cap was screwed down and the pressure rise followed with the strain gauge. Any initial leaks were immediately detected by a drift in the strain gauge pressure. With the cap in place and a proper seal obtained initial pressure reading was 2400 lb/in^2. Experience has shown that for reproducible results, this exact initial pressure must be obtained. Virus control samples were placed in similar plastic tubes and attached to the exterior of the steel cell which was then placed at the appropriate freezing temperature. After the desired pressure was reached the cell was returned to room temperature and the presure fall followed until it reached 2400 lb/in^2 at which point the cell was opened and the samples removed. The strain gauge is very sensitive to slight fluctuations in pressure. Moreover, the cell pressure will vary with any changes in freezer temperature. Thus, opening and closing the freezer door while pressure is being developed will change the shape of the pressure curve. The pressure curve is directly related to temperature at which freezing occurs and Fig. 1 shows the characteristics of pressure curves at −12 and −22°C, the temperatures used in the present experiments. With this particular apparatus maximum attainable pressure is about 32,000 lb/in^2. After this pressure is reached a fall in pressure occurs if the cell is allowed to remain at the same temperature, generally to about 27,000 lb/in^2. Finally, under the present experimental conditions, it is not possible to hold a pressure between 2500 and 27,000 lb/in^2 once it is attained.



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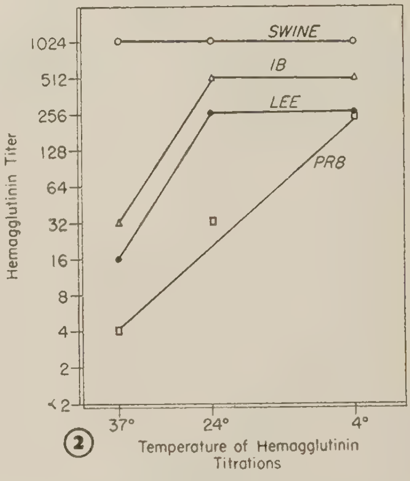

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PRESSURE = 25,000 Lbs/in^2

FIG. 1. Relation between pressure and temperature at which cell is filled with water and virus samples is placed.

FIG. 2. Relation of titer of virus subjected to pressure and temperature at which hemagglutinin test was performed.

Results. Initially, all pressure experiments

TABLE I. Effects of Pressure Developed at –22°C and –12°C on Influenza Virus Hemagglutinin.

Influenza virus strains	Virus hemagglutinin titers at 24°C						
	Lb/in^2 pressure developed at –22°C					Lb/in^2 pressure developed at –12°C	
	15,000*	17,000	20,000	24,000	30,000	15,000	17,000
SW	512	512	512	512	512	512	512
PR$_8$	”	”	”	64	<2	”	”
FM-1	”	256	128	128	256	”	128
Lee	”	”	”	”	”	”	64
IB	”	512	512	512	512	”	128
1233 (‘‘C’’)	1024	1024	1024	1024	1024	1024	1024

* Titers equivalent to control samples of virus not subjected to pressure.

were performed by placing the cell at –22°C. Allantoic fluids containing virus strains described were diluted 1:2 in normal saline and subjected to pressures ranging from 2500 lb/in^2 (the baseline pressure) to 30,000 lb/in^2. At end of run, control and pressurized fluids were removed and hemagglutinin titered at room temperature. The results are given in Table I. One of the most striking features has been the reproducibility of the data. Thus, in replicate experiments amount of decrease in hemagglutinin titers for any given strain at any given pressure was always the same. Considering individual results, there is a marked difference in effect on hemagglutinin from various influenza virus strains. Thus, although the SW and PR8, strains of type A influenza virus are related rather closely antigenically, there is a vast difference in susceptibility to inactivation by pressure of these hemagglutinins. In other experiments, SW virus was subjected to a peak pressure of 32,000 (later falling to 27,000) for 4 days without effect on the virus hemagglutinin. Likewise, no effect of pressure has been found with the 1233 strain of influenza "C" virus. The FM-1, Lee, IB and BCal strains were variably affected by pressures developed at –22°C. The slight (one tube) rise in hemagglutinin titer in FM-1 and Lee upon increasing pressure from 24,000 to 30,000 lb/in^2 probably is not significant. In addition to strains described, a few studies with mumps, Newcastle Disease virus and NWS showed no pressure inactivation of the hemagglutinin. Since addition of serum tends to protect virus from inactivation by various physical methods, serum was added to virus fluids to be tested in the pressure cell. No serum concentration tested (the highest was equal quantities of allantoic fluids and horse serum) showed any protective effect. Due to a mechanical failure of one freezer unit it became necessary to conduct the experiments with the cell at a much higher temperature, –12°C. This, of course, resulted in more prolonged rise in pressure. For example, at –22°C a pressure of about 16,000 lb/in^2 can be obtained in about 4 hours whereas at –12°C 16 or more hours are required to attain the same pressure level. Unfortunately, the highest pressure obtainable at this temperature is only about 19,000 lb/in^2. The results of pressure experiments obtained at this temperature also are presented in Table I. Surprisingly, the Lee, FM-1, and IB virus hemagglutinins were more affected under these conditions than at presures more rapidly attained in the previous experiments.

Effect of temperature of titration on hemagglutinin titers of virus subjected to pressure. Since hemagglutinin was affected by pressure it was of interest to determine the role of virus elution in this process. Accordingly, the virus strains subjected to pressures with the cell at –12 and –22°C were then titered at temperatures of 4, 24, and 37°C. The results of these experiments are presented in Table II and Fig. 2. In general, virus samples subjected to various pressures showed a decrease in titer as the titration temperature was raised. Moreover, in certain instances the higher temperatures demonstrated a pressure effect on the hemagglutinin not apparent with room temperature titration. Thus, IB virus compressed to 17,000 with the cell at –23°C had a titer equivalent to the control when titered at 24°C but showed a 4-fold decrease at 37°C. Again, the difference in effect on the Lee, IB and

TABLE II. Titer of Influenza Virus Hemagglutinin at 24°C and 37°C of Virus Subjected to Pressures Developed at −22°C and −12°C.

	Lb/in^2 pressure developed at −22°C				Lb/in^2 pressure developed at −12°C	
	17,000 lb/in^2		24,000 ib/in^2		17,000 lb/in^2	
	Temperature of hemagglutinin titrations					
Influenza virus strains	24°	37°	24°	37°	24°	37°
SW	512	512	512	512	512	512
PR$_8$	"	"	64	16	"	"
FM-1	256	64	128	<2	128	<2
Lee	"	128	"	16	64	"
IB	512	"	512	32	128	64

FM-1 hemagglutinin when rate of increase of pressure was varied was apparent. Lee virus for example brought to 17,000 lb/in^2 pressure (−23°C) showed a 4-fold decrease at 37°C titration temperature yet if the same pressure were slowly attained and the sample then titered at 37°C the resultant decrease was more than 128-fold. To determine more precisely the relationship between titer and temperature of titration various virus strains were subjected to 25,000 lb/in^2 pressure and titered at 37°, 24°, and 4°C. The results of this experiment are shown in Fig. 2. The titer of the control samples in this experiment was 512 for IB, Lee and PR8 and 1024 for the SW strains. Obviously, there is a relationship between temperature of titration and titer of the IB, Lee and PR8 strains but this is not always proportional. Again, no effect on SW hemagglutin was found.

Infectivity and antigenicity inactivation of influenza virus by pressure. Since the effect of pressure on hemagglutinin varied with the strain of virus used it was of interest to determine whether such variation could be demonstrated with infectivity titration. The results of experiments on 3 strains are shown in Table III. Whereas no pressure affected the SW hemagglutinin, a pressure of 24,000 lb/in^2 (1600 atmospheres) almost completely inactivated the infectiousness of this strain. Moreover, this pressure resulted in loss of most of the infectivity of the other 2 strains of virus tested.

TABLE III. Effect of Pressure Developed at −22°C in Infectivity of Influenza Virus.

	Pressure, lb/in^2			
	15,000		24,000	
Influenza virus strains	Control	Exp.	Control	Exp.
PR8	7.5*	7.5	7.5	2.5
SW	7.8	7.6	7.6	1.4
Lee	7.8	7.2	7.5	1.0

* Log ID$_{50}$ in eggs.

The PR8 virus was used in immunization studies to determine whether infectivity and/or hemagglutinin could be destroyed by pressure without affecting the antigenicity. For this purpose virus was semipurified by differential centrifugation and the virus then divided into control and pressure-treated lots. After 25,000 lb/in^2 pressure the resultant virus suspensions showed a marked loss of antigenicity when tested in rabbits. Rabbits immunized with pressurized virus developed an antihemagglutinin titer of 1:80 compared with the control titer of 1:1280 in animals immunized with the same aliquot sample not subjected to pressure.

Electron microscope studies of PR8 virus particles subjected to maximal pressures have not shown detectible changes in particle morphology.

Discussion. The present experiments indicate a marked difference between strains of influenza virus in the effect of pressure on viral hemagglutinin. The variation in resistance to presure inactivation is particularly marked with the swine as compared to the PR8 strains of type A influenza virus. Thus, maximal pressures for periods as long as 4 days failed to affect SW hemagglutinin yet more than 90% of PR8 hemagglutinin was lost at lower pressures applied for much shorter intervals. Other physical agents such as heat(4) and ultraviolet irradiation(5) have also demonstrated some differences between influenza virus hemagglutinins of the strains tested. However, the differences were most apparent in comparison of type A and type B viruses and, to our knowledge, no studies previously reported have shown such marked differences in influenza virus hemagglutinins closely related antigenically.

As might be anticipated from other experiments concerned with physical agents and influenza virus, inactivation of infectivity and hemagglutinin does not occur coincidentally. Part of the aim of the present experiments was to determine whether pressure inactivation might be useful as a technic to prepare killed-virus vaccines. Unfortunately, the results indicate that antigenicity is lost at pressures requisite for infectivity inactivation so that, at least with the influenza virus strains tested, pressure inactivation for vaccine preparation would not appear feasible.

Summary. High compression pressures attained at below freezing temperatures have been studied for their effects on influenza virus hemagglutinin, infectivity and antigenicity. Influenza virus hemagglutinin from the virus strains tested varies markedly in its resistance to pressure inactivation.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Separation of Lymphocytes in Human Blood by Means of Glass Wool Column.* (25237)

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With the exception of the magnetic technic of Levine(1), previous procedures for separation of lymphocytes from the other white cells found in human blood(2,3) have been based on differences in density of the various formed elements. This report describes the successful application of the principle of differential adsorption to separation of human lymphocytes. In this technic, granulocytes are removed from whole blood by passage through a short column of siliconized glass wool, while the lymphocytes in good yield and apparently intact are recovered in the effluent.

Materials and methods. The column and attachments are shown in Fig. 1. All glass surfaces were siliconized with a mixture of monomethyltrichloro- and dimethyldichlorosilane.[‡] The vapor of this mixture (obtained by forcing air through a bubbler) was passed through the glass wool for 1 minute and then quickly flushed out with air. The glass wool was washed 10 times with water, heated 30 minutes at 110°C, soaked in water 30 minutes, rinsed many times with distilled water, and finally air dried. The glass wool used was Pyrex fiber No. 3950.[§] Two grams of glass wool were packed into the column and held under firm pressure overnight by a rubber stopper fitted with a glass rod. The column was first conditioned by passing through it about 10 ml of fresh serum under 5% carbon dioxide, 85% nitrogen, 10% oxygen. Preliminary experiments carried out since the series reported in this paper indicate that this conditioning may be unnecessary. A freshly drawn 30 ml sample of the same subject's blood was then obtained, heparinized with 3.0 mg of heparin, and added to the column. No attempt was made to control the gas phase over the whole blood. The flow rate was adjusted to about 0.8 ml/minute by turning the screw clamp on the vent tube. The first 4 ml of effluent blood were discarded, and the next

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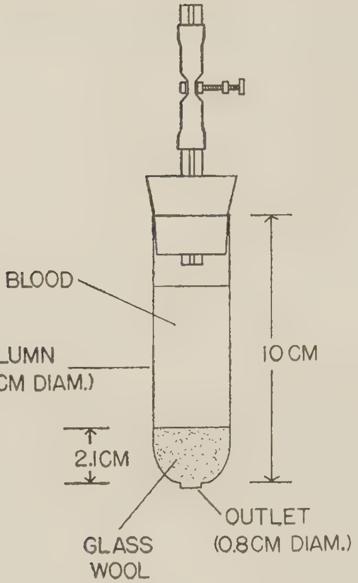
* Supported by Grants from Nat. Inst. of Health, and by William Edris Fund.

[†] SF-238, Nat. Inst. of Health, U.S.P.H.S.

[‡] Dow-Corning 1208, New Products Dept., Dow-Corning Corp., Midland, Mich.

[§] Corning Glass Works, Corning, N. Y.



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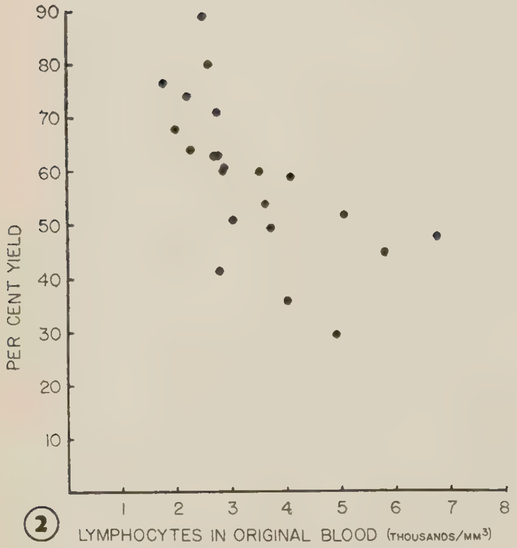

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FIG. 1. Diagram of glass wool column.

FIG. 2. Relationship between % yield* of lymphocytes and total lymphocyte count in original blood samples.

$$* \frac{\text{Lymphs. in 24 ml of effluent blood}}{\text{Lymphs. in 30 ml of original blood}} \times 100 = \%$$ yield.

TABLE I. Cellular Composition of Blood before and after Passage through a Column of Siliconized Glass Wool.

	Original blood	Effluent blood
Total white blood count (thousands/mm³)	5.3-10.6* 8.2†	1.8-4.3 2.6
% lymphocytes	24-62 41	67-100 90
% polymorphonuclear neutrophils	35-72 57	0-33 9.8

* Range † Avg

24 ml were collected for study. The effluent, which consisted essentially of whole blood minus the granulocytes, was examined for its cellular composition. All differential counts were made from smears on chemically clean glass slides and stained with Wright's stain. Two hundred cells were counted in the first 11 experiments and 100 cells were counted in the second 11 experiments. In counting lymphocytes a category of "distorted" lymphocytes was included although such forms may be seen regularly in smears of normal blood. This category of lymphocytes included cells with the following alterations: 1) cytoplasm partially pulled away from the nucleus, 2) nucleus markedly elongated of deformed, and 3) cytoplasm present only in wisps or leaves about the nucleus. Degenerated cells were infrequent and not enumerated.

Results. The results of 22 consecutive experiments using blood from normal human adults are summarized in Table I. Removal of the granulocytes was somewhat more effective than suggested by the table since in 12 of these experiments the proportion of lymphocytes in the effluent blood was 95% or higher. Platelets were seen in abundance in the effluent smears but no quantitative measurements were made. Using a water-jacketed column, there was no difference between results obtained at 27°C and 37°C. However, at 16°C and 4°C large numbers of granulocytes were found in the effluents.

The integrity of the lymphocytes obtained in these effluents was judged primarily upon morphological bases. Ten "distorted" lymphocytes were found in counting 954 lymphocytes in the smears from original blood for a ratio of 1.05 "distorted" lymphocytes per 100 lymphocytes counted. Twenty-four "distorted" lymphocytes were found in counting 2009 lymphocytes in smears from effluent blood for a ratio of 1.15 "distorted" lymphocytes per 100 lymphocytes counted. The small difference between the 2 groups supports the impression that lymphocytes were relatively undamaged in their passage through the column. Typical smears from effluents were examined by a hematologist,‖ and were judged to be normal in all respects. Phase contrast microscopy of lymphocytes in fresh blood and effluent blood also showed no detectable differences in appearance. Per cent yield of lymphocytes was inversely related to absolute lymphocyte count in the original blood (Fig. 2).

Discussion. This procedure offers promise as a means of preparing lymphocytes for metabolic study, especially since no colloidal or other foreign matter is required to remove the granulocytes. Blood can be processed quickly to remove granulocytes, then one of the methods for removal of erythrocytes can be applied.

The mechanism by which the granulocytes appear to be adsorbed upon the glass wool is unknown. The temperature dependence suggests that the cells must be metabolically active, but it is not clear whether some further features of the interaction of the cell surface with the adsorbant are involved. A count of band form neutrophils in both original and effluent bloods was made in the first 11 experiments. No band forms were found in any of the effluent bloods although from 0-6% were found in the original bloods. This suggests that the young forms are no less sticky than the more mature ones.

The tendency for a greater proportion of lymphocytes to be retained on the column when the original blood has a high lymphocyte count is also unexplained. Several of the subjects with high original lymphocyte counts had just recovered from upper respiratory infections. This relationship to previous disease is currently being studied.

Summary. The method of differential adsorption has been successfully applied to separation of lymphocytes from granulocytes in human blood. When whole blood was passed through a short column of siliconized glass wool, the granulocytes were almost quantitatively retained on the column, and uninjured lymphocytes in 30-89% yield appeared in the effluent blood.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

‖ We are indebted to Dr. Walter A. Rambach, Dept. of Hematology, Northwestern University Medical School for making these evaluations.

Effect of Endotoxin on Plasma Catechol Amines and Serum Serotonin.* (25238)

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The exact role of the pressor amines in endotoxin shock has not been clearly defined, though it has often been observed that systemic effects of endotoxin appear to be associated with generalized sympathomimetic responses. The work of Zweifach, Nagler and Thomas led to the conclusion that an abnormal response to the catechol amines may be an important factor in the lethal effects of endotoxin(1). However Meyer and Ballin have been unable to confirm this finding(2). The effects of serotonin (5-hydroxytryptamine) are far more obscure. Gordon and Lipton demonstrated a protective effect of 5-hydroxytryptamine (5-HT) in endotoxin shock and later pointed out an "antagonism" between this substance and noradrenaline(3,4). Haddy *et al.* showed that serotonin has a vasodilatory effect on small vessels and a vasoconstrictor effect on large vessels(5). In situations where these vessels are maximally constricted, the net effect is vasodilatation.

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* Aided by research grant from Graduate School, Univ. of Minnesota.

TABLE I. Effect of Endotoxin on Plasma Catechol Amines and Serum Serotonin.

	Dogs surviving less than 12 hr								
	Epinephrine (μg/l)			Norepinephrine (μg/l)			Serotonin (μg/ml)		
	Control (mean)	After 5 min.	Terminal	Control (mean)	After 5 min.	Terminal	Control (mean)	After 5 min.	Terminal
	.16	65.81	89.80	2.58	36.48	21.98	1.12	.13	.63
	1.82	.00	180.72	2.48	32.80	49.26	.48	.01	.24
	.00	.10	90.81	.00	30.33	27.28			
	1.44	20.14	457.20	.24	18.00	92.87	.47	.19	.28
	3.46	55.40	287.70	2.36	19.10	84.00	.43	.10	
	.91		144.75	2.92		55.40			
	.67		53.10	4.76		9.85	.24		.14
Mean	1.21	28.29	186.30	2.19	27.34	48.66	.55	.11	.32

This might account for the protective effect of 5-HT in endotoxin shock. More recently, it has been shown that serum serotonin decreases following administration of endotoxin(6) and that a marked depletion of serotonin stores occurs as part of the anaphylactic reaction (7). These findings might explain an altered response of blood vessels to epinephrine and norepinephrine on the basis of a serotonin deficiency. These studies were designed to determine change in concentration of plasma catechol amines and serum serotonin following administration of endotoxin in order to help clarify the role of these substances in producing the aforementioned effects.

Materials and methods. Mongrel dogs weighing approximately 20 kg were sedated with morphine and given a lethal dose (7.5-10 mg/kg) of *E. coli* cell suspension intravenously.† Venous blood samples were obtained before administration of endotoxin and at regular intervals during the shock state. Blood loss incurred by sampling was replaced. The following determinations were performed; plasma catechol amines by Weil-Malherbe's method(8), serum serotonin by Davis' method(9), platelet count and hematocrit.

Results. It was found that the plasma catechol amine concentration generally reflected the blood pressure, *i.e.*, when the latter fell, concentration of epinephrine and norepinephrine (particularly the former) rose. The plasma catechol amines usually increased immediately after administration of endotoxin, simultaneously with the initial sudden fall in blood pressure (Table I). By 30 minutes, concentrations approaching control levels were found, and they remained unchanged until the animal began to deteriorate and the blood pressure began to fall. Extremely high values were obtained when the animal was in extremis (Table I). A marked fall in serum serotonin concentration occurred following administration of endotoxin (Table I) which persisted throughout the shock state. There was a tendency for the concentration to recover somewhat as time progressed. In an animal which survived the experiment normal levels were reached. However, in those that succumbed, 5-HT fell back to the level it had attained immediately after endotoxin was administered.

Discussion. These studies would indicate that endotoxin can incite the release of toxic amounts of epinephrine into the circulating blood. It seems, however, that the extremely high levels which are attained become manifest after the animal has suffered metabolic and physiologic derangements which produce its eventual demise. The significance of the initial elevation of plasma catechol amines is hard to evaluate. During most of the interval between administration of endotoxin and death, plasma catechol amine concentration is only slightly elevated. Serum serotonin, however, is always far below its pre-shock level. In view of the mean decrease in serotonin to 18.3% of the control level, the elevated levels of epinephrine and norepinephrine may have a physiologic effect greater than their concentrations would indicate. The fall in serotonin concentration is consistent. We have also found it to occur in rabbits following administration of a lethal dose of endotoxin. The question of how these changes are produced by endotoxin remains to be answered. Since anaphylactic reactions have also been shown to be accompanied by low levels of 5-HT in the blood and a generalized sympathomimetic response, the above data further demonstrate the similarity between these two phenomena (10).

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† Generously supplied by Dr. W. Spink, Dept. of Medicine, Univ. of Minnesota Hospitals.

Summary. Elevated levels of epinephrine are found immediately after administration of a lethal dose of endotoxin. Following this, plasma catechol amine concentration is only slightly above control levels. Prior to death, toxic levels of epinephrine are attained. Serum serotonin concentration falls immediately after administration of endotoxin and remains low throughout the shock state.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Variation with Age of Antistreptococcal Antibodies and Their Correlation With Antistreptolysin O Titers in Human Sera.* (25239)

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Halbert, Swick and Sonn(1) found that sera from non-rheumatic individuals gave from 0-4 bands, when tested by Ouchterlony's technic(2,3), against a crude ammonium sulfate concentrate of the supernate from a Group A streptococcus culture (C 203 S). Sera from active cases of rheumatic fever gave 2 to 7 bands when similarly tested. Harris, Harris and Ogburn(4) obtained similar results using Oudin's method(5) and the concentrated supernate of a type 4 culture. Halbert *et al.*(6) also observed a correlation between migration rate of the heaviest bands, antistreptolysin O (ASO) titers of the sera and intensity of the precipitate supposedly produced by the ASO system. This study is concerned with the immunogenic response to streptococcal antigens of normal individuals of different ages in a tropical environment. Possible correlations between precipitins, as evidenced by gel diffusion, and ASO titers have been investigated.

Materials and methods. Human sera comprised 9 taken from the umbilical cord of infants at birth, 74 from infants 1 to 12 months old, many of whom were suffering from diarrheal disease, and 331 from apparently normal adults aged 17 to 70 years, selected randomly at various factories, blood banks and public health dispensaries. The antigen used consisted of a type 3, group A, beta hemolytic streptococcus inoculated into 500 ml of trypticase soy broth and incubated at 37°C for 18 hours. Culture was divided into 50 ml portions, lyophilized and each dissolved in 5 ml of sterile saline just before use. Same batch of lyophilized material was used in all gel diffusion tests(1). Usually antigen was placed in the center and sera in the peripheral wells. Plates were placed in the refrigerator and precipitin bands read at intervals of 3, 7, 14 and 21 days. Antistreptolysin O assay was done with Bacto Streptolysin O reagents (Difco) and results expressed in Todd units.

Results. All sera from umbilical cord blood revealed the presence of 1 to 3 bands with an

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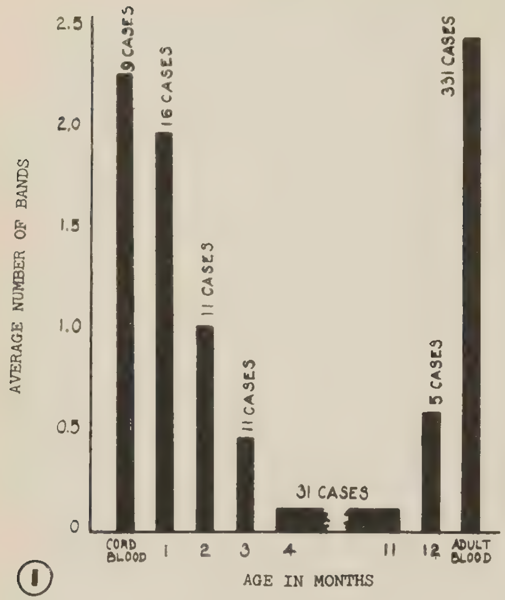
* Supported by grant from Nat. Heart Inst., N.I.H., U.S.P.H.S.



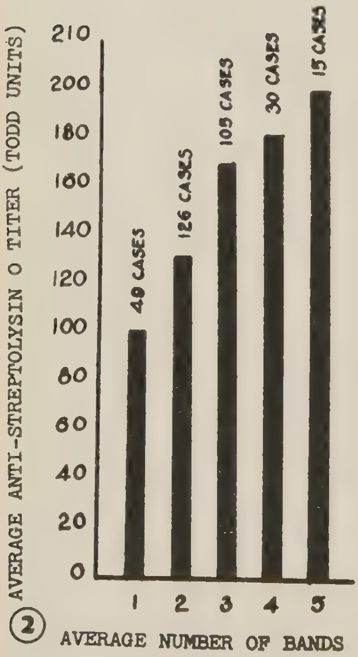


FIG. 1. Fluctuation with individual's age in the No. of bands (gel diffusion) in sera tested with a streptococcus whole culture concentrate.

FIG. 2. Correlation between ASO titers and No. of bands (gel diffusion) in adult sera.

average of 2.2 bands per individual. A sharp drop in the number of bands was observed in infants 1 to 5 months old (Fig. 1). Sera from 29 out of 31 infants 4 to 11 months old were free from demonstrable anti-streptococcus antibodies. At 12 months the average number of bands per infant rose again to 0.6. The number of bands in 331 adults ranged from 0 to 5 with an average of 2.45 per individual which is about the same value obtained with cord sera. Forty-five adult sera gave 4-5 bands. This was not observed in any of the sera from infants in which the maximum number of bands was 3.

Findings in infant sera can probably be explained on the basis of transplacental transfer. These antibodies are short-lived and disappear from the infant's blood during the first 3 to 5 months of life. They reappear upon encounter with the streptococcus later in life. No significant fluctuation obtained in the average number of bands in individuals 17 to 70 years old.

Results suggest the desirability of a simultaneous study of the antibody pattern of mother and infant in an attempt to determine selective transplacental transfer of the different streptococcal maternal antibodies.

We did not perform ASO determinations in these infants' sera. There are reports in the literature of ASO determinations in infants which correlate with our observations(7). Three hundred thirty-one adult sera were assayed for ASO and by gel diffusion methods. Sera were classified into 5 groups according to number of bands obtained (from 0 to 5). Results are given in Fig. 2.

Good correlation obtains between number of bands and ASO titer which is not apparent when individual cases are considered. Average ASO titer for sera showing a single band was 100.6 units. ASO titers rose gradually in almost regular steps with each increase in number of bands. It is difficult to interpret the observed correlation between ASO titers and number of bands, representing a single antigen-antibody and a multiple antigen-antibody system respectively. The correlation found by Halbert, between migration rate of the ASO band and ASO titer is to be expected, as it has been established that rate of migration of a given band depends on respective concentrations of antibody and antigen(5). Our findings suggest that when ASO titer increases there is a concomitant stimulation of antibody response to multiple streptococcal antigens elaborated *in vivo*. It seems that the more recent the date of the last streptococcal infection suffered by the patient, the stronger the antibody response observed. Apparently each antibody has a threshold level which has to be reached before antibody makes itself evident as a band by gel diffusion. Recent streptococcal infection could bring several of these antibodies to their threshold value resulting in an increase in number of bands and a concomitant increase in ASO titer. As time passes, concentration of antibody decreases with a corresponding diminution in number of bands and titer of the ASO system. The fact that a greater number of bands is obtained with the sera of rheumatic fever patients, in whom high ASO titers occur, seems to corroborate this interpretation of results.

Using a crude concentrated streptococcal supernate, Halbert found 0 to 4 bands in a series of 35 non-rheumatic individuals with an average of 1.51 bands per individual. We found an average of 2.45 bands. This may be due to our use of antigen which contained streptococci, from which cellular antigens could presumably be liberated by disintegration of the bacterial cell. Halbert(6) reports a surprising infrequency of bands with sonic vibrated cell extracts, as well as with individual intracellular constituents such as C carbohydrate and M substance.

It is interesting to note the almost complete absence of negative results (only one case) among adults, in contrast with the 31% of negative sera in Halbert's series. He believes that many of their apparently negative sera may have had bands masked by a nonspecific turbidity of the serum-agar gel. In our experiments this interference was minimal. We observed that all adult sera, with the exception already mentioned, produced a band located approximately midway between the antigen and antibody wells, which gave reactions of identity with similar bands in adjacent sera, with the formation of a hexagonal pattern. Other bands were more variable in position and frequency.

An attempt was made to determine degree of contamination of our antigen with C carbohydrate and M substance. This was done by comparing our preparation with antigenic preparations consisting of (a) hydrochloric acid extract of streptococci prepared by the method of Lancefield(8). (b) autoclaved extract of streptococci prepared by the method of Rantz and Randall(9). (c) C carbohydrate prepared by the method of Fuller(10). The 4 antigens were tested side by side against commercial Bacto anti-A serum. The last 3 antigens gave a prominent, well defined band, with reaction of identity with each other. Our antigen gave an extremely faint and diffuse band with reaction of identity with the other three. This indicates an extremely small contamination of our preparation with these intracellular antigens. In addition, we tested several normal sera against extracts a, b and c with completely negative results.

Summary. 1. Sera from newborn infants were examined by gel diffusion against whole culture concentrate of type 3, Group A beta hemolytic streptococcus. One to 3 bands of precipitation were obtained with average of 2.2 bands/individual. In the first 3 to 4 months of life the average number of bands/individual diminished sharply to almost complete absence. From 4 to 11 months of age the average number of bands/individual was 0.13. On twelfth month the average number/infant rose to 0.6, and in adults an average of 2.45 was obtained. These results are interpreted on the basis of transplacental transfer of antibodies which are short-lived and reappear with subsequent streptococcal infection later in life. 2. Sera from 331 apparently normal adults were studied by the gel diffusion method and simultaneously assayed for antistreptolysin O. A close correlation was found between number of bands and ASO titers, not apparent when individual cases were compared. With each increase in number of bands there was a corresponding increase in ASO titer.

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Received July 15, 1959. P.S.E.B.M., 1959, v102.

Influence of Resting Tension on Contractility and Morphology of the Papillary Muscle.* (25240)

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The papillary muscle preparation has become a popular tool in the field of cardiac research. A wide variety of factors, *e.g.*, resting tension, manner of recording, composition of bathing media, have been studied with respect to their effects on contractility or morphology. A review of the literature discloses only 4 publications(1-4) in which resting tension was stated as an absolute value. Bennett(5) made a detailed study of the effect of tension on physiological response but did not examine the muscles for any associated morphologic changes. Tanz *et al.*(6,7) reported alterations in morphology of papillary muscles exposed to drugs, but failed to indicate the resting tension to which their preparations were subjected. In a preliminary report(8) experiments were described in which a study was made of the relationships between initial resting tension, stability of initial contractile amplitude or tension, and morphology of working papillary muscles. The present report includes studies on nonworking as well as working muscles.

Methods. Cats were anesthetized with ether, the heart removed, and placed in warm, oxygenated, modified Tyrode's solution of the following composition: (meq/l) Na, 145.00; K. 5.34; Ca, 4.75; Mg, 1.20; Cl, 129.55; PO_4, 1.79; SO_4, 1.20; HCO_3, 25.00; glucose, 5.0. A thin papillary muscle, 1.0 mm or less in diameter, was selected from the right ventricle and ligatures were placed around it at its junction with the ventricular wall and the chorda tendineae. The muscle was then removed from the ventricle and placed in a muscle holder, care being taken not to apply tension to the muscle. The holder was immersed in a bath containing the previously described Tyrode's solution maintained at a temperature of 37.5°C and aerated by a rapid stream of 95% oxygen and 5% carbon dioxide entering through a sintered glass disk in the bottom of the bath. A supramaximal stimulus of 1 to 2 millisec in duration was provided as a square wave from an American Electronic Laboratories, Inc., Model 751, stimulator at the rate of 1/sec. In isotonic preparations, the upper ligature was attached to a light heart lever adjusted to give the desired resting tension and record on a smoked drum. For isometric studies, the ligature was attached to the pin of a RCA 5734 transducer tube in a bridge circuit similar to that described by Ranney(9). This arrangement allowed the transducer to be calibrated with weights and the resting tension to be adjusted to that desired. A total of 30 muscles were studied, the tension ranging from 1.6 to 4.0 g. Some muscles were merely subjected to static tension and not stimulated. The muscles were removed from the bath after 6½ hours and fixed in 10% formalin. After fixation the muscles were dehydrated and embedded in paraffin. Sections approximately 5 micra in thickness were cut and stained with hematoxylin and eosin.

Results. Muscles subjected to tensions of

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* This investigation supported in part by grant from Wisconsin Heart Assn.

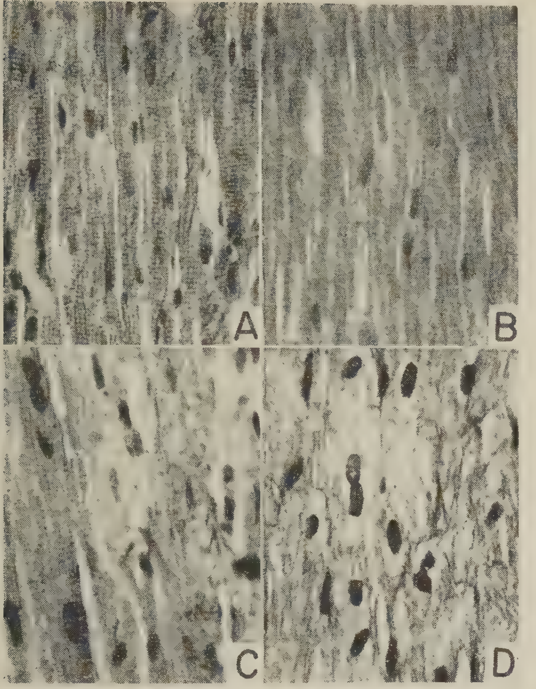

FIG. 1. Effect of loading on histologic appearance of papillary muscle of the cat. A. Fresh tissue, B. 1.6 g load, C. 3 g load, D. 4 g load. Loaded muscles were fixed following 6½ hr of isotonic contraction.

less than 3.0 g. (Fig. 1B) were morphologically similar to those (Fig. 1A) fixed immediately after removal from the animal. After being subjected to a tension of 3.0 g, muscles exhibited areas of early degeneration interspersed with areas of normal morphologic appearance (Fig. 1C). After application of 4.0 g. of tension, severe degeneration was evident (Fig. 1D). Warped and pycnotic nuclei and foamy cytoplasm were present and striations were absent. Work did not appear to be a significant factor in determining degree of morphologic change since muscles which were not stimulated to contract showed essentially the same morphologic alterations as working muscles subjected to an equal amount of tension. However, the degenerative changes noted in isotonic preparations appeared to be more pronounced than those observed in isometric preparations under the same conditions of tension and activity.

In isotonic preparations, contractile amplitude and degree of failure varied considerably, depending on tension applied. If the muscles were loaded with 1.6 g, contractile amplitude decreased slowly and in a fairly predictable manner. The results from several experiments are shown in Fig. 2. When tension was increased to 3.0 g, it was not possible to predict the course of the contractile amplitude. Some preparations with a resting load of 3.0 g behaved similarly to those loaded with 1.6 g while others failed rapidly, displaying a reduction of contractile amplitude of as much as 80% by the end of experiment. Lack of a uniform response was due, perhaps in part, to a variation in size of the individual muscles. Under a 4.0 g load, failure was rapid and complete within 2 hours.

Under isometric conditions papillary muscles displayed similar contractile characteristics with loads ranging from 1.6 to 4.0 g. Increasing the load from 1.6 to 4.0 g did not significantly affect rate or degree of failure. Fig. 2 depicts the average response for all muscles with the range of loads employed.

Discussion. The results of this investigation show that resting tension is important with respect to both contractile and morphologic characteristics of the papillary muscle preparation. Bennett(5) reported that muscles contracting isotonically with a load of 2.0 g exhibit a contractile response similar to that shown in Fig. 2 for muscles loaded with 1.6 g. Gold and Cattell(3) showed that after a short period, the contractile amplitude of the papillary muscle under 1.2 g of isometric tension stabilized and thereafter decreased very slowly

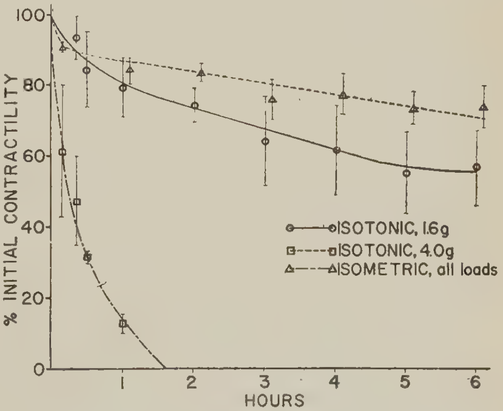

FIG. 2. Effect of loading on contractility of papillary muscle of the cat. Vertical lines represent stand. errors for each mean value. Each curve represents avg of results from 3 or more preparations.

over a period of 5 hours. Deterioration of contractility is much more rapid in a medium containing phosphate rather than bicarbonate as a buffer(4,10).

Our experiments indicate that isotonically contracting preparations of the papillary muscle are quite sensitive to the load applied and that the speed with which failure ensues is a function of this load. On the other hand, failure in isometric preparations does not appear to be correlated with tension. If one is interested in the effects of drugs, *e.g.*, the cardiac glycosides, on mechanically produced failure in the papillary muscle the type of preparation and degree of loading perhaps are not critical as long as these variables are maintained constant. However, if one wishes to examine the effects of drugs on a preparation which approximates the normal or non-failing heart, it appears that resting tension is an important factor and should be taken into consideration. These experiments also show that tension alone can result in morphologic changes, ranging from slight alterations to severe degeneration. The mechanism of this degeneration is not clear but such changes are not unique in as much as they are observed in tissue post mortem if fixation is not carried out rather promptly.

Summary. Papillary muscles of the cat subjected to various resting tensions exhibited morphologic changes, the severity of which could be correlated with degree of loading with loads of 3 g or greater. These changes were similar to those which occur post mortem in cardiac muscle and appeared whether the muscle was stimulated to contract or subjected to static tension. Morphologic alterations appeared in both isotonic and isometric preparations but appeared more severe in isotonic preparations. Rate and degree of failure of isotonically contracting muscles could be correlated with size of the resting load but such a correlation was not observed with muscles contracting under isometric conditions.

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Received July 17, 1959 P.S.E.B.M., 1959, v102.

Thermal Sensitivity of Polioviruses Isolated during Oral Vaccine Field Trial: Comparison with Monkey Neurovirulence.* (25241)

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Recent observations by Dubes *et al.*(1,2), Lwoff and Lwoff(3), and Sabin and Lwoff(4), indicated that virulent strains of poliovirus grow readily at temperatures of 39° and 40°C, while multiplication of attenuated strains is greatly reduced at such relatively high temperatures. In the following experiments an effort was made to determine if this characteristic, here designated the thermal marker, T (for attenuated), I (for intermediate), and T^+ (for virulent), could be used for the fol-

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* Aided by grant from Nat. Foundation.

† Fellow of James Hudson Brown Memorial Fund, Yale Univ. School of Med.

lowing purposes: 1) to detect any reversion to greater virulence of an attenuated poliovirus vaccine strain which might occur as a result of passage in the human intestinal tract; and 2) to determine whether the thermal marker could be applied as a screening test for differentiating poliovirus strains recovered in the field with respect to their neurovirulence, and thus might aid in differentiating the attenuated LSc strain of type I poliovirus from various other strains isolated during a field trial in which LSc had been introduced into the community as an oral vaccine. The results with the thermal marker were also compared with another *in vitro* marker, *viz.*, the capacity to multiply at low and high bicarbonate concentrations, the "d-d^+" character of Vogt, Dulbecco, and Wenner(5), and with neurovirulence as determined by intracerebral inoculation of rhesus monkeys.

Materials and methods. Virus strains. Strains of type I poliovirus isolated during an oral vaccine field trial using Sabin's LSc type I attenuated poliovirus were tested.‡ The trial was carried out in a village community near Phoenix, Ariz., and details concerning virus administration and collection and testing of specimens have been reported (7). Virus isolates tested were strains from the intestinal tracts of 3 children fed and infected with the LSc strain; one strain from the privy of an infected family; and 15 strains obtained from flies trapped at various points in and directly adjacent to the study area and also peripherally in the village. For comparison, 2 control poliovirus strains were used, i) virulent Mahoney type I strain, and ii) attenuated type I LSc vaccine strain from an aliquot of the same pool orally administered to children in the trial. In addition, examples of virulent and attenuated types II and III polioviruses were also tested for comparison. These were: —MEF 1 (virulent) and YSK (attenuated) for type II; Leon (virulent) and Leon KP_{34} (attenuated) for type III.§ Monkey kidney (MK) monolayer cultures were used throughout(8). Bottle cultures were overlaid with agar as described by Hsiung and Melnick(9). For determination of growth curves, of Mahoney and the LSc strains, tube cultures were used. *Tests for the thermal marker.* Second, or in some instances third, MK passage of strains to be tested were inoculated in 10 fold dilutions into 3 sets of MK bottle cultures, 2 bottles/dilution. After one hour's adsorption at 37°C, the cultures were overlaid with agar. One set was then incubated at 35°C, another at 37°C, and a third at 39°C. The 37°C incubator used was a walk-in type, employed routinely, while the 39°C and 35°C ones are specially constructed units each with a fan and thermostat (Fenwal thermoswitch) intrinsically sensitive to 0.1°F temperature changes. The temperatures in these 2 incubators had been recorded on a thermograph continuously over 3 weeks before experiments were begun, and at intervals thereafter; the daily variation was less than 0.5°C. During tests, the 35° and 39°C incubators were not opened until 4-5 days of incubation had been completed. At this time plaque titers/0.1 ml were determined. The efficiency of plating (EOP) at 37°C was taken as unity, the EOP of cultures grown at 35° and 39°C being related to this standard. In each test the attenuated LSc type I strain which had been fed to children in the field trial and the virulent Mahoney type I strain were included as controls. *Growth curves of type I strains.* Virulent Mahoney and attenuated LSc strains of type I virus were each inoculated into 3 sets of 24 MK tubes in doses of 2 x $10^{5.5}$ TCD_{50}/ml. During adsorption period of 1 hour, the cultures were held at 37°C. Following this, cultures were washed 3 times with balanced salt solution, the medium was replaced and 24 tubes of each set were incubated at 35°, 37°, and 39°C respectively. Every 2 hours for the first 12 hours of incubation, and at 24 and 48 hours, 3 tubes held at each temperature were frozen and thawed once, the harvests pooled and titrated for total virus in MK tubes. At time of harvesting, cultures were examined for cytopathogenic effect (CPE). *Other tests for virulence and genetic stability.* In most instances, the same inocula were used both in testing for

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‡ We are grateful to Dr. Albert B. Sabin for making the LSc type I oral vaccine(6) strain available to us.

§ Attenuated types II and III strains kindly provided by Dr. Albert B. Sabin(6).

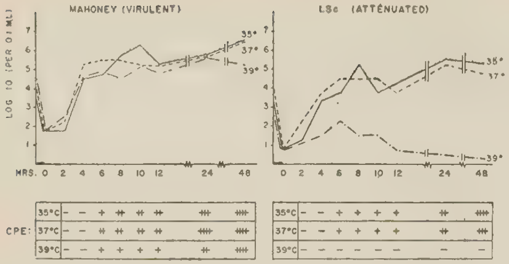

FIG. 1. Growth curves of virulent and attenuated type I poliovirus strains at different temperatures. Plus signs in panels at base of charts indicate degree of cytopathic effect (CPE).

thermal marker and for other virulence tests; in a few, the same passage level but a different preparation was employed. *The "d" marker* was determined in agar overlay cultures with low (0.08%) and high (0.5%) bicarbonate concentrations, as described by Hsiung and Melnick(10). *Monkey neurovirulence* was tested by inoculating a minimum of one million TCD_{50} of each strain intracerebrally into 2 rhesus monkeys. The dose was contained in 1 ml, of which 0.5 ml was introduced into each side of the thalamus. Animals were observed daily for 3-4 weeks. Those which developed signs of poliomyelitis were sacrificed at the height of paralysis, while the others were sacrificed at the end of the test period. The medulla and 3 levels of spinal cord were examined histologically for lesions of poliomyelitis.

Results. Growth curves of Mahoney and LSc type I strains at different temperatures. Fig. 1 indicates that 2 distinct patterns emerged, one for the virulent Mahoney strain, the other for attenuated LSc type I strain. Each point on curves represents a titration of harvests of 3 tube cultures. The Mahoney strain multiplied equally well at 35°, 37°, and 39°C, whereas multiplication of the LSc strain was greatly reduced at 39°C. Since all cultures were held at 37° for the adsorption period of 1 hour, the first cycle growth of LSc at 39° probably represents virus which adsorbed at 37°C and multiplied to a limited extent during the shift to 39°C.

Cytopathic effect (CPE) was observed at all 3 temperatures with the Mahoney strain, beginning at 6 hours (Fig. 1). With the LSc strain, no CPE was observed at 39°, although it appeared at 6 hours at both 35° and 37°C.

EOP of virulent and attenuated strains of polioviruses types I, II, and III at different temperatures. Virulent strains of all 3 types each had a high EOP at 39°C, while the attenuated strains all showed a low EOP at this temperature (Table I). The figures for EOP are expressed as log of the quotient when the titer (PFU/0.1 ml) at 39° (or 35°) is divided by the titer at 37°C. The results of several different tests are shown in Table I.

Thermal marker of type I strains isolated during LSc oral vaccine field trial. Comparison with other virulence tests. Results are shown in Table II. The virus isolated from 2 of the children who had received the oral vaccine, #23-8 and #20-8, exhibited the same pattern with respect to thermal marker as did the ingested LSc virus. The third child, #10-5, 4 days after LSc was orally administered, also excreted virus with a comparably low EOP at 39°C, but by 14th day, the excreted virus showed a shift to a slightly higher EOP at this temperature, and by the 29th post-feeding day virus isolated from this child had an EOP at 39°C characteristic of virulent strains. The d-d^+ marker of these same strains exhibited a less regular pattern, but the one T^+ strain was also d^+. Although all 7 strains isolated from the infected children were relatively avirulent for the monkey, a million or more TCD_{50} of

TABLE I. Growth of Virulent and Attenuated Polioviruses at Different Temperatures.

				Efficiency of plating			
			Titer in PFU	at 39°C		at 35°C	
	Type	Strain	per 0.1 ml at 37°	EOP	Log	EOP	Log
I	Virulent	Mahoney	8.5×10^6	.41	− .39	.64	−.2
	Attenuated	LSc	4.0×10^5	.000025	−4.6	.75	−.1
II	Virulent	MEF_1	2.0×10^6	.2	− .7	1.5	+.2
	Attenuated	Y-SK	4.5×10^4	.000011	−4.96	4.0	+.6
III	Virulent	Leon	8×10^6	.5	− .3	.7	−.2
	Attenuated	" KP_{34}	$>7.5 \times 10^5$	<.00013	−3.9	>7.3	+.9

TABLE II. Results of Virulence Tests on Type I Poliovirus Strains, Isolated during a Field Trial with Attenuated LSc Strain Type I Oral Vaccine. Comparison of 3 markers.

Source of poliovirus	Strain No.	Date of collection* (1958)	Virulence tests: Thermal (T) marker† (EOP at 39°C)		"d" marker‡ (EOP at low bicarbonate conc.)		i-c monkey inoc.§ (dose 10^6-10^7 TCD_{50})		
			Log EOP	Marker	Log EOP	Marker	Paralysis	Lesions	Marker
Excreted by infected children‖	23-8	17/II	–5.5	T	–3.2	d	0/2¶	0/2¶	M–
	"	24/II	–5.3	T	–3.0	i	"	"	"
	20-8	18/II	–6.0	T	–4.8	d	"	"	"
	"	20/II	–6.0	T	–1.9	i	"	"	"
	10-5	17/II	–6.1	T	–3.8	d	"	1/2	Mi
	"	27/II	–4.1	T	–1.9	i	"	"	"
	"	14/III	–1.3	T+	– .6	d+	"	"	"
Privy of infected family	20-A	20/II	–4.0	T	–4.3	d	"	"	"
Flies trapped in village	10-01	20/II	–4.8	T	–2.0	i	"	0/2	M–
	6-01	27/II	–3.5	I	–3.8	d	"	2/2	Mi
	1-26	20/III	–3.4	I	–2.2	i	2/2	"	M+
	"	19/II	–3.2	I	– .9	d+	1/2	"	"
	10-01	19/II	–2.8	I	–3.7	d	"	"	"
	13-03	25/II	–2.4	I	–1.5	i	2/2	"	"
	1-26	20/II	–2.4	I	–2.5	i	1/2	"	"
	"	27/II	–2.2	I	–2.0	i	2/2	"	"
	7-13	27/II	–1.9	T+	–2.0	i	"	"	"
	4-14	18/III	–1.7	"	–1.0	d+	"	"	"
	6-01	11/III	– .9	"	–1.7	i	"	"	"
	"	6/III	–1.8	"	–1.6	i	"	"	"
	3-06	20/III	– .7	"	–2.1	i	1/1	1/1	"
	4-17	18/III	– .4	"	–1.5	i	2/2	2/2	"
	3-06	11/III	+ .1	"	–1.5	i	"	"	"
Control virulent	Mahoney (virulent)		– .9	"	– .5	d+	(10/10)**	(10/10)**	"
			– .3	"	– .3	"			
			– .6	"	– .2	"			
and			– .3	"	– .5	"			
			– .4	"	– .4	"			
Attenuated type I strains	LSc (attenuated)				–4.2	d	0/9	0/9	M–
			–6.0	T	–4.0	d			
			–5.4	T	–5.8	d			
			–6.2	T	–5.0	d			
			–5.8	T	–4.2	d			

* LSc type I fed to 6 children in community 12/II/58.

† Designation "T" marker as follows: Log EOP $\leqq -4.0$ = T (attenuated).
-2.1 to -4.0 = I (intermediate).
> -2.0 = T+ (virulent).

‡ Designation "d" marker: Log EOP < -3.0 = d (attenuated).
-3.0 to -1.0 = i (intermediate).
≥ 1.0 = d+ (virulent).

§ Designation as to monkey neurovirulence: Neither lesions or paralysis = M– (attenuated). Lesions but no paralysis = Mi (intermediate). Lesions and paralysis = M+ (virulent).

‖ 23-8, 20-8, and 10-5 refer to 3 LSc fed children who became infected.

¶ Denominator indicates No. tested, numerator, No. positive.

** Tests performed earlier in this laboratory.

virus failing to produce signs of weakness or paralysis in any, CNS lesions were present in one of 2 animals inoculated with each of the 3 specimens isolated from child #10-5.

Strain isolated from privy of infected family. Strain #20-A (Table II), the only strain of poliovirus isolated from a fecal sample obtained from a privy(7), possessed the T and d markers, and induced no paralysis in 2 out of 2 inoculated monkeys, but one of the animals showed mild lesions.

Strains isolated from flies. Fifteen strains, isolated during field trial from 7 different trapping sites, had thermal marker patterns ranging from the T, characteristic of attenuated strains, to the T⁺ of virulent strains. Unfortunately, no flies were collected before the trial began, so that strains isolated prior to introduction of the LSc strain into the community were not available for comparison. Only one of the fly strains isolated approached the range of the LSc strain fed: this was #10-01, collected 20/II, which had an EOP of log —4.8, and failed to produce either paralysis or lesions in the 2 monkeys inoculated. The EOP of this strain at low bicarbonate concentrations placed it in the intermediate (i) range with respect to the d-d⁺ marker. Seven other fly strains were characterized as intermediate in thermal sensitivity tests; all possessed the I thermal marker, and produced lesions in 14 animals inoculated, as well as some degree of muscle weakness in 9 of them. With respect to the d-d⁺ marker, 2 of these 7 strains were classified as d, 4 as intermediate, and one as d⁺ (Table II). Of the remaining 7 fly strains, all were T⁺ with respect to the thermal marker, and all produced paralysis and lesions in 13 of 13 monkeys inoculated. Comparing the T-T⁺ and d-d⁺ markers, the 7 T⁺ strains were characterized as d⁺ in one instance, and as intermediate (i) in 6.

Discussion. Results of growth curves with virulent and attenuated type I poliovirus strains at different temperatures confirm the observations of others(1-4) that reduced multiplication at high (39°C) temperatures is a characteristic of attenuated poliovirus strains. This is further borne out by the EOP of plating at 39°C found for virulent and attenuated strains of types II and III polioviruses (Table I).

However, the results also indicate the difficulty in identifying a poliovirus strain isolated in the field as one which has been introduced into the community, even when 2 different *in vitro* and one *in vivo* "virulence" tests were employed. Nevertheless, our findings suggest that the thermal marker is probably a more reliable measurement than is the d-d⁺ marker in this respect. It correlated better with monkey virulence tests in which the intracerebral route of inoculation and large doses were used, and gave more consistent results with strains isolated from persons *known* to be infected with a given strain, in this instance the LSc strain of attenuated type I virus.

As Table II shows, only one of the 15 fly strains behaved as did the LSc virus ingested. This strain, #10-01, 20/II, was isolated from flies trapped in the study area near a family in which a child had received the vaccine virus and become infected. It had a very low EOP at 39°, and failed to produce either paralysis or lesions in intracerebrally inoculated monkeys. It may have been a wild attenuated strain; or on the other hand, it may have been the LSc strain of virus which had been picked up by the local flies; this latter occurrence would not be surprising, considering that there were children in the area known to be infected with the LSc strain, and the level of sanitation (7) provided flies with ready access to fecal material.

Of the remaining 14 fly strains tested, 7 were intermediate (I) strains and 7 were T⁺ with respect to the thermal marker. Many of these strains in all probability represent naturally occurring wild strains, some of lower and others of a higher degree of virulence both by *in vitro* and *in vivo* tests. Nevertheless, one cannot completely rule out the possibility that some strains might be the LSc, somewhat enhanced in virulence after human passage. That many strains were isolated from flies trapped early in the course of the trial, at widely separated points far from the 3 infected families, makes it seem more likely that they represent wild strains. Furthermore, the range of their T markers and monkey neurovirulence are similar to the patterns which we have found for *known* wild type III poliovirus strains isolated at the same time from the same traps.

From these results, one can conclude that the thermal marker is helpful in characterizing freshly isolated strains as relatively virulent or attenuated; further it is an aid in following the course of events after feeding attenuated poliovirus to humans, in determining whether enhancement of virulence occurred, and perhaps to some extent, whether the environment is contaminated with the vaccine virus. Particularly is this true if the T marker of a given strain is considered in connection with other markers such as the d-d$^+$, and monkey neurovirulence. However, if the community under study is one in which poliovirus infections are highly endemic and naturally occurring strains of low virulence are prevalent, the problem of strain differentiation by means of above markers becomes complex. In such a situation thermal and other markers can suggest the identity of a given strain but cannot provide positive proof. It is possible that more clear cut results and better correlation of the T marker with monkey neurovirulence could be achieved if a level of 40°C rather than 39°C is used as the critical temperature. This possibility is currently under investigation.

Summary. 1. Virulent and attenuated strains of the 3 types of poliovirus have been shown to have different patterns of growth at high (39°C) temperatures when tested by means of agar overlay tissue culture technic. Attenuated strains (T marker) are inhibited and have a low EOP at this temperature, while virulent strains (T$^+$) grow equally well at 35°, 37°, and 39°C. Some strains give an intermediate (I) pattern. 2. The thermal marker T-T$^+$ was used to screen poliovirus strains isolated during a type I oral vaccine field trial, in an effort to determine: a) whether reversion to greater virulence had occurred as a result of human passage; and b) whether strains isolated from local flies were the vaccine virus which had been orally administered, or were naturally occurring wild strains. The results indicated that virus excreted by 2 of the 3 infected children consistently had thermal markers identical with that of the vaccine virus; one child, on the other hand, excreted virus which over a period of 29 days showed a shift to the thermal marker pattern of more virulent strains. Neurovirulence for monkeys was low for all strains isolated from children. One T strain isolated from flies had an EOP at 39°C in the same range as the LSc vaccine strain, while the 14 other fly strains tested all fell between this range and that of virulent strains. It was concluded that most of the fly strains recovered in the course of the field trial probably represented naturally occurring wild strains. 3. Comparison was made between the thermal (T-T$^+$) marker, the EOP at low and high bicarbonate concentrations (d-d$^+$ marker), and neurovirulence as tested by intracerebral inoculation of monkeys. The thermal marker correlated better with monkey virulence than did the d-d$^+$ marker. 4. Use of the thermal *in vitro* marker should be of some practical value in determining degree of spread of attenuated poliovirus strains introduced into a community, and in assessing degree of stability of vaccine strains on human passage.

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Received July 17, 1959 P.S.E.B.M., 1959, v102

In vitro Transfer of Radiocadmium, CD^{115}, from Plasma to Human Erythrocytes.* (25242)

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A finding incidental to earlier studies(1,2) in this laboratory was *in vivo* transfer of radiocadmium, Cd^{115M}, from plasma to canine erythrocytes following intravenous administration of the isotope. This report concerns further investigation of the kinetics of cadmium metabolism, specifically with respect to *in vitro* transfer of the metal from plasma to erythrocytes. Such studies are particularly appropriate since cadmium is related to enzymatic phenomena(3,4), is found in trace amounts in man(5,6), and has been reported to occur naturally in the equine kidney cortex(7).

Materials and methods. Cd^{115} (half life, 53 hours) was obtained from Oak Ridge National Laboratories as cadmium nitrate in excess nitric acid and neutralized with sodium hydroxide before use. Absorption studies indicated the presence of a small amount of Cd^{115M} (half life, 43 days) as a contaminant. The latter did not interfere with the present investigation, however. Heparinized blood was obtained from donors, and after 30 minutes, 30 ml were placed in paraffinized flasks, suspended in a constant temperature bath (38°C) with continuous agitation. Approximately 10 μc of Cd^{115} were added to each flask and thoroughly mixed with the contents. After one, 2, 4, 8, 12, and 24 hours, samples were withdrawn from the flasks. The blood samples were centrifuged in Wintrobe hematocrit tubes, and supernatant plasma was removed for counting. The buffy coat was removed, and residual erythrocytes washed 3 times with human Ringer's solution before counting to determine rate of transfer of Cd^{115} to the erythrocytes. To detect hemolysis, the serum potassium was determined by the flame photometric technic in each sample. All counting was done with a thin mica end window Geiger-Muller counter, using methods previously described(8).

Results. The results are illustrated in Fig. 1, in which black circles represent specimens in which hemolysis occurred, and white circles represent non-hemolyzed specimens. Except for subject 6, the erythrocytes generally behaved in like manner. Samples collected through the eighth hour were free from detectable hemolysis. Thereafter varying degrees of hemolytic activity were noted in all specimens. Thus, the similarity of results obtained in later specimens may represent incomplete erythrocyte binding of cadmium due to loss of erythrocytic binding substance to the serum. Conversely maximal metal uptake might have occurred prior to hemolysis with subsequent release of bound cadmium. Further studies are needed to determine these points.

In a single subject (No. 6) prompt and marked binding of cadmium occurred. Degree of binding persisted at essentially the same level in spite of later hemolysis, suggesting that such bonds, once formed are relatively stable. The patient from whom this sample was obtained was the only member of the group with a hematologic abnormality. During the 2 weeks prior to sampling, he suffered from considerable gastrointestinal bleeding, secondary to a malignant process. It is inviting to postulate that increased cadmium uptake was due to the presence of an increased number of circulating immature red blood cells as a result of the bone marrow stimulation arising from hemorrhage. The latter have a higher concentration of nucleic acid, and a stickier surface, but less globin. At this time there was no obvious explanation for the increased affinity of this subject's erythrocytes for cadmium.

Discussion. The erythrocyte is rich in constituents with which cadmium is capable of forming compounds of varying stability(9,10). Since there exists a broad spectrum of similar affinities, the qualitative and quantitative aspects of such unions are complex and largely

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* Aided by U. S. Public Health Service grants.

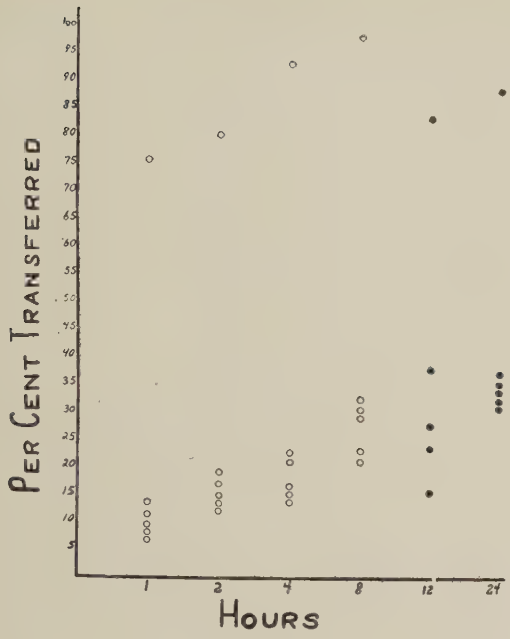

FIG. 1. % Cd^{115} transferred from plasma to human erythrocytes.

unknown. Without the application of more sophisticated technics, it is impossible to determine whether or not cadmium formed a stable complex with the constituents of the red cell surface or entered the erythrocyte proper. The former seems unlikely, particularly since similar studies with radioactive sodium chromate have indicated that most of the test isotope was bound to intracorpuscular globin, and only insignificantly to hematin (11). Nevertheless, the numerous metabolic transfer systems of the erythrocyte require localization to the cell surface of enzymes which may bind cadmium to a variable degree. Many intracorpuscular substances and enzymes are capable of uniting with cadmium. Perhaps, as suggested for mercury, cadmium principally combines with thiols. Other reactions may involve amino, hydroxy, imidazole, or carboxyl groups.

Summary. The 24-hour in vitro transfer of radiocadmium, Cd^{115}, from plasma to human erythrocytes was investigated. Most of the isotope became erythrocyte-bound during the first 8 hours of incubation although subsequent kinetics were somewhat obscured by hemolysis. The magnitude of the shift approximated 30% of total cadmium, although the erythrocytes of one subject, who had recently hemorrhaged, exhibited a much greater affinity for the metal. These findings suggest the desirability of further studies to determine the value of cadmium as an indicator of erythrocyte survival.

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Received July 20, 1959. P.S.E.B.M., 1959, v102.

Comparison of Physiological Properties of Certain Phage-typable Coagulase-positive Staphylococci.* (25243)

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Although the literature on physiological activities of Staphylococci is extensive, little has been published concerning a comparison of these properties between different phage-typable groups. The purpose of our studies

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* Supported in part by U.S.P.H.S. Grant.

TABLE I. Biological Activities of Certain Strains of *Staphylococcus pyogenes*.

	Phage type			
	(a) 44A	(b) 44A/80/81	(c) Other	(d) Non-typable
	16 strains	31 strains	9 strains	34 strains
Free coagulase	%			
a. Tube	100	100	100	100
b. Glycine-tellurite	”	”	”	”
Bound coagulase	”	94.7	”	78.2
Pigment (orange)	”	89	”	96.8
Mannitol	”	100	”	100
Delta lysin	68	16	77.1	73
Alpha ”	81	81.1	100	89
Beta ”	6	3.2	11	28
Fibrinolysin	18.7	25	11.7	50
Phosphatase	100	100	100	100
Urease	18.7	.0	.0	23.5

was to determine whether there was any correlation, other than coagulase production, of biological activity and phage type.

Materials and methods. Ninety strains were collected of freshly isolated coagulase-positive staphylococci from human pyogenic infections. These organisms were phage typed, through the courtesy of Dr. J. V. Irons, director, Texas State Health Laboratory, Austin, according to the standard procedure. For our purpose the staphylococci were divided into 4 groups as follows: Those lysed by a) phage 44A, b) by both phages 44A and 80/81, c) by one or more phages other than 44A or 80/81, and d) those not lysed by any of the 25 standard phages. Ability of staphylococci to form "free" coagulase was measured by 2 procedures: The tellurite-glycine method of Zebowitz *et al.*(1) and standard test tube dilution procedure employing fresh rabbit plasma and supernatant of 24 hour culture of staphylococci grown in brain-heart infusion broth. Bound coagulase was measured by the slide method in accordance with Cadness-Graves *et al.*(2).

Alpha hemolysins as defined by McFarlan (3) were measured on 5% rabbit blood agar. Beta hemolysins were quantitated by determining their activity on sheep red blood cells, Smith and Price(4), and the culture filtrate heated to 55°C for 30 minutes thus destroying rabbit cell lysins and leaving about 50% of original beta lysin intact. Delta lysin activity was measured on human red blood cells after Marks and Vaughan(5).

Fibrinolysin activity was determined by procedure of Neter(6). The presence of the enzyme urease was tested for using urea broth as outlined by Fusillo and Jaffurs(7). Phosphatase activity was measured by splitting of phenolphthalein from phenolphthalein diphosphoric acid in agar, see Hare and Thomas (8). Pigment production was assessed on Staph 110 agar (Difco) following 48 hours incubation at 30°C. Ability to utilize mannitol as source of carbohydrate was measured in mannitol broth (Difco) incubated 48 hours at 37°C.

Results. Table I shows the correlation of tube-plasma mthod of determining free coagulase and the tellurite-glycine agar was excellent. It further appears that it measures free coagulase and not bound. All free-coagulase-positive staphylococci produced phosphatase and utilized mannitol and the majority produced the characteristic golden pigment.

In accordance with observations of Elek and Levy(9) it appears that beta lysin production is not common to strains of staphylococci from human sources. Although 28% of non-typable group produced some sheep blood hemolysin, titers of activity rarely exceeded the 1:2 dilution. Close correlation of alpha lysin production with coagulase is a well-known fact. It, however, cannot be a sole criterion for pathogenicity as all our strains were isolated from a pathologic process and some failed to produce alpha lysin. We were unable to confirm the observation of Joiris(10) that human strains of staphylococci producing alpha lysin almost invariably produce delta lysin. Only 16% of Group b (phage type 44A/80/81) produced both hemolysins.

It is of some interest that 50% of Group d (non-typable) strains exhibit fibrinolytic activity. This value is considerably higher than the 14% reported by Neter(6). While Fusillo and Jaffurs(7) defined a relationship between activity of coagulase and inactivity of urease among strains of staphylococci, our observations showed some urease activity in strains of Group a (44A) and Group d (non-typable).

Summary. Free coagulase can be measured adequately by either the tube-plasma method or the tellurite-glycine method. We were unable to establish any direct relationship between certain groups of phage-typable staphylococci and production of alpha, beta or delta hemolysins. Fibrinolytic activity was variable but 50% of Group d (non-typable) exhibited some activity.

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Received July 22, 1959. P.S.E.B.M., 1959, v102.

Lowered Glucose Utilization, Phosphate Uptake and Reduced Glutathione of Erythrocytes Following Uretero-Caval Anastomosis.* (25244)

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Following bilateral nephrectomy of the dog, glucose utilization, phosphate uptake and reduced glutathione content of erythrocytes are significantly lowered(1). Bilateral nephrectomy is also associated with a prominent shortening of the life span of erythrocytes(2). Anastomosis of one ureter into the inferior vena cava followed by contralateral nephrectomy is attended by the same degree of retention uremia as after bilateral nephrectomy, yet the life span of erythrocytes is significantly improved toward normal during uretero-caval anastomosis(3). These findings have suggested protection against *in vivo* hemolysis by intact renal tissue(3). With this background it became of interest to determine the state of glucose utilization, phosphate uptake and reduced glutathione content of erythrocytes in the uremia of the dog following uretero-caval anastomosis.

Material and methods. Normal mongrel dogs were used. Under nembutal anesthesia one ureter was severed near the urinary bladder and connected to the inferior vena cava by means of a polyethylene tube. Through a purse string suture about a puncture of the vena cava the polyethylene tube was extended for 3 to 5 cm along the blood stream. Three to 4 days later the contralateral kidney was removed during ether anesthesia. Control blood samples were obtained prior to nephrectomy. The test samples were obtained 4 days following removal of the contralateral normal kidney. After the test sample was obtained the animal was sacrificed and the patency of the ureter and tube and state of the kidney were determined. In 5 of the 7 experiments the ureter and tube were open; there was no hydronephrosis and the kidney was of normal

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* Supported by grant from Nat. Heart Inst., U.S.P.H.S.

TABLE I. Changes in Glucose Utilization, P-32 Uptake and GSH Content of Erythrocytes following Uretero-Caval Anastomosis. Control value was taken prior to removal of normal kidney. Degree of azotemia is given in last column.

					GSH content (mg/100 ml R.B.C.)					
	Glucose utilization (mg/100 ml R.B.C.)		P-32 uptake (%)		Nitroprusside method		Alloxan method		Plasma urea (mg/100 ml)	
Wt, kg	Control	4 days	Control	4 days	Control	4 days	Control	4 days	Control	4 days
* 9.1	38.1	46.9	34.1	24.9	57.6	39.8	57.9	41.5	76	486
10.0	63.8	34.6	43.0	29.4	55.8	37.3	59.8	37.3	72	386
* 8.4	51.5	38.1	37.5	22.8	61.7	33.2	61.4	46.3	74	366
15.0	55.4	31.2	35.6	26.9	66.2	41.7	74.4	42.8	82	402
11.0	56.0	32.9	41.3	31.6	55.3	28.6	54.7	28.8	100	468
9.1	58.5	29.8	42.1	33.4	57.9	44.8	58.3	47.6	86	394
9.1	42.5	39.3	37.0	30.1	48.7	42.1	48.9	41.9	65	330
Mean diff.	−16.75		−10.21		−19.4		−18.5			
p *vs* o	.02		.001		.001		.001			

* Obstructed ureters. All other examples had non-obstructed ureters.

configuration. In 2 instances the polyethylene tube was obstructed by blood clots. The anastomosed kidneys increased in weight over the contralateral kidney by an average of 48% over the 4 days. This marked hypertrophy has been shown to be associated with an increase in normal appearing cortical and medulla tissues(4). These observations indicated an adequate circulation through the kidney with the uretero-caval anastomosis. Duplicate analyses on each sample revealed minimal variations. The values were averaged. Ten ml of blood were obtained from the femoral artery into a syringe containing a drop of heparin. The samples were immediately chilled in ice and analyses were begun shortly thereafter. For the glucose utilization the Somogyi glucose method(5) and the general approach described by Hollingsworth(6), were used. The inorganic phosphate uptake was conducted by the method described by Prankerd(7). The reduced glutathione content was obtained by 2 methods, the nitroprusside method of Grunert and Phillips(8) and the alloxan method(9). The plasma urea concentration was determined by an urease method(10).

Results. The data and their statistical analyses are given in Table I. The Table contains paired data, that is the control values after uretero-caval anastomosis but before removal of the contralateral normal kidney and the values 4 days after the nephrectomy. During the 4 days the ureter was connected to the vena cava and there was no excretion of urine to the outside. A "t" test was used to determine whether or not the mean difference between the control and the 4 days values differed significantly from zero.

The results from all 3 measurements differed significantly from zero indicating a lowering of all 3 biochemical measurements. There was no significant difference in the reduced glutathione values as obtained by the alloxan and Grunert and Phillips methods. While these results were derived a pronounced azotemia accrued.

Comment. The 3 biochemical measurements of erythrocytes used (glucose utilization, phosphate uptake, reduced glutathione content) were lowered to the same extent in the presence of intact hypertrophying renal tissue without an excretory outlet as following renal ablation. Since uretero-caval anastomosis and bilateral nephrectomy are associated with a similar degree of retention uremia, the findings suggest that either hypertrophying renal tissue cannot prevent these red cell changes or that the changes are more related to the uremia *per se*. The resolution of these two possibilities was not possible by this study.

Bilateral nephrectomy of the dog is associated with a pronounced shortening of the life span of erythrocytes. Since the uretero-caval procedure as used herein changes the erythrocytic life span significantly toward normal(3), the present study does suggest that the biochemical measurements used are not as directly related to the shortened erythrocytic life span as was previously considered (1).

Summary. 1. Glucose utilization, phosphate uptake and reduced glutathione content of erythrocytes are significantly lowered following uretero-caval anastomosis and contralateral nephrectomy of the dog. 2. Above biochemical changes are of the same magnitude as following bilateral nephrectomy. Thus presence of hypertrophying renal tissue without an excretory outlet is not protective toward these alterations.

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Received July 23, 1959. P.S.E.B.M., 1959, v102.

Influence of Vitamin A on Cholesterol Blood Levels. (25245)

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There is evidence that a relationship exists between elevated serum cholesterol levels and atherosclerosis(1,2). It therefore seemed worthwhile to investigate further substances which might be capable of reducing serum cholesterol levels. Vitamin A* was chosen because of favorable reports of its antihypercholesteremic properties in animals. Weitzel, Schon, and Gey(3) reported that Vit. A had a definite protective effect on experimental atherosclerosis in rats. Later, the same workers(4) gave large oral doses of the fat-soluble Vit. A, E, and K to old atherosclerotic hens, over a period of 75 to 100 days. With Vit. E, a slight antiatherosclerotic effect was observed in a few cases. With Vit. A, however, macroscopic examination showed marked regression of the atheromatosis in all the test groups and a decrease in total fat content of the aorta. However, Oppenheim and Bruger,(5) reported that oral doses of Vit. A did not alter the pattern of development of experimental atherosclerosis in rabbits. Wendt(6) reported that massive doses of Vit. A caused an increase in cholesterol content of human serum. Lasch (7) reported that Vit. A, given as "Vorgan" 3 times a day to human beings, in doses of 40,000 to 80,000 I.U. caused an increase in serum cholesterol within 5 to 10 days. This increase was due to an increase in serum cholesterol esters.

Method. Two types of patients were used in this investigation: first, normal individuals, aged 30 to 50; and second, individuals having experienced a myocardial infarction within the year, aged 40 to 55. The above groups were subdivided; those receiving Vit. A and those receiving no medication. Thus there were 4 different experimental groups. *Group A.* Each of these patients experienced at least one myocardial infarction within the year. All individuals showed elevated serum cholesterols, total, free, and ester levels. 100,000 I. U. of Vit. A acetate per day was administered orally to each patient. *Group B.* Each of these patients had suffered at least one myocardial infarction within the year. All had elevated cholesterol levels, total, free, and esters. No medication was administered to this group. *Group C.* All patients had normal cholesterol levels, total, free, and esters. No clinical history of coronary artery disease. 100,000 I. U. of Vit. A acetate per day was administered orally to

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* Vit. A was furnished by Hoffman-La Roche, Nutley, N. J.

TABLE I. Average Blood Levels of Groups A, B, C, and D.

Groups	No. of patients	Total cholesterol, mg % Before therapy	Total cholesterol, mg % After therapy	Vit. A, μg % Before therapy	Vit. A, μg % After therapy
A	8	355 ± 51*	275 ± 24	56 ± 8	107 ± 29
B	5	318 ± 59		59 ± 22	
C	5	240 ± 24	236 ± 15	51 ± 38	101 ± 15
D	5	230 ± 28		69 ± 22	

* Stand. dev.

each patient. *Group D.* As in Group C, all patients had normal cholesterol levels, total, free, and ester. No clinical history of coronary artery disease. No medication was given to this group. In Groups A and B, serum was obtained every 2 weeks for 6 months, and in Groups C and D, for 3 to 4 months. All patients were requested to restrict their fat intake. Average dietary intakes are given in Table II. Weekly diet charts were recorded at intervals during the investigative period. Cholesterol determinations, total, free, and esters, were carried out on all patients in triplicate, biweekly, by the method of Schoenheimer and Sperry(9). With these patients in Groups A and C, several determinations were made to establish a mean cholesterol level before initial Vit. A therapy. Vit. A determinations were carried out monthly on all patients using the Carr-Price antimony trichloride method(8).

Results. Following administration of Vit. A, all of the atherosclerotic patients (Group A), showed a decrease in total cholesterol, (Table I) ranging from 20 mg % to 175 mg %, which was attributed to a decrease in the ester fraction. The greatest reductions were seen in patients with the highest initial levels. Free cholesterol remained constant in all cases.

No significant change was seen in the cholesteral levels, total (Table I), free and ester in any of the other experimental groups.

Vit. A levels determined on all patients were either within normal limits or slightly elevated, before initiation of Vit. A therapy. Patients in Groups A and C who received supplementary Vit. A exhibited an average Vit. A blood level of 107 ± 28.6 and 101 ± 15.2 μg % respectively 4 to 6 months after vitamin therapy was initiated (Table I). All patients tolerated 100,000 I.U. of Vit. A acetate without untoward symptoms.

As shown in Table II, about 20% of dietary intake in all patients was fat, and daily caloric intake was approximately 2,300 calories.

Discussion. The effect of Vit. A therapy on cholesterol levels in patients suffering from atherosclerosis of varying degrees, (Group A) was encouraging. Average total cholesterol blood level in this group before therapy was 355 ± 50.9 mg % and 275 ± 24.4 mg % after therapy (Table I). This difference was shown to be statistically significant. Statistically no significant change was seen in the other experimental groups.

Since the accuracy for the Schoenheimer-Sperry procedure for cholesterol determination ranges between 10-15 mg %, part of the deviation seen in each group may be attributed to limitations of the method.

A small percentage of the deviation in all groups might be attributed to diet but this cannot be considered too significant since all patients ingested a similar diet.

Although little is known about mode of action or site of action of this vitamin in the body, it would seem that one of its actions is to affect elevated serum cholesterol levels. No significant effect was noted in Group C in

TABLE II. Average Daily Dietary Intakes.

Group	CHO, g	Fat, g	Fat, %*	Protein, g	Table calories
A	253 ± 98†	69 ± 70	19 ± 11	83 ± 86	2298 ± 624
B	276 ± 52	80 ± 69	20 ± 8	91 ± 33	2412 ± 219
C	237 ± 43	75 ± 52	18 ± 7	101 ± 71	2300 ± 416
D	266 ± 49	98 ± 75	21 ± 11	91 ± 75	2410 ± 612

* % fat calculated on basis of percentage of total 6 ms. carbohydrate, protein, and fat.
† Stand. dev.

which the patients had normal cholesterol levels. It might be postulated that Vit. A either inhibits synthesis of cholesterol in cases of over-synthesis, or causes mobilization and excretion of cholesterol when blood levels become excessively high.

These results are in direct disagreement with work done by Wendt(6) and Lasch(7). Their reported increase in serum cholesterol after Vit. A administration may be explained on the basis of the interference of Vit. A with cholesterol determination as pointed out by Kinley and Krause(10). Since Lasch and Wendt were using a reagent containing ferric chloride which was used both for cholesterol determinations and Vit. A determinations, their apparent elevations were possibly due to a color produced by both cholesterol and Vit. A. The same type of elevation was seen in this laboratory until the Schoenheimer-Sperry method was adopted for separation of Vit. A from the serum before carrying out cholesterol determinations.

Summary. Oral administration of 100,000 I. U. of Vit. A acetate for 4 to 6 months significantly reduced the elevated serum cholesterol levels in atherosclerotic patients but had no effect on individuals with normal cholesterol levels.

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Received July 24, 1959. P.S.E.B.M., 1959, v102.

Further Studies of Gaseous Ion Action on Trachea.*† (25246)

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During the past 3 years, it has been demonstrated that light air ions markedly affect the functional efficiency of the mammalian trachea(1,2). In general, (+)ions depress ciliary activity, contract the tracheal wall and induce a state of vasoconstriction and hyperreactivity to mechanical trauma. (−)ions accellerate the rate of ciliary beat, reverse the (+)ion-induced contraction of the wall, and do not alter normal vascularity or the response to trauma. Experiments performed to determine which atmospheric components in ionized form account for the observed functional changes implicated negatively charged oxygen and positively charged carbon dioxide respectively as the specific mediators for acceleration and inhibition of ciliary activity (3). However, these observations apply to only one physiological effect, ciliary activity. Because experiments on other effects, such as enhanced vulnerability to trauma, require exposure of the trachea *in situ* to nitrogen and carbon dioxide for long periods of time, a procedure not compatible with life, a method was devised which permitted the animal to respire through a separate airway while response of the trachea to treatment with various gases was observed.

Methods. 1) Operative technic: Albino rabbits weighing 10-12 lb were anesthetized

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* This research was supported by contracts with: (1) Atomic Energy Comm. and (2) U. S. Air Force (A.F. 49 (638)-669) and by grant from Committee on Research, Univ. of California.

† The opinions expressed herein are not necessarily those of the Air Force or Navy.

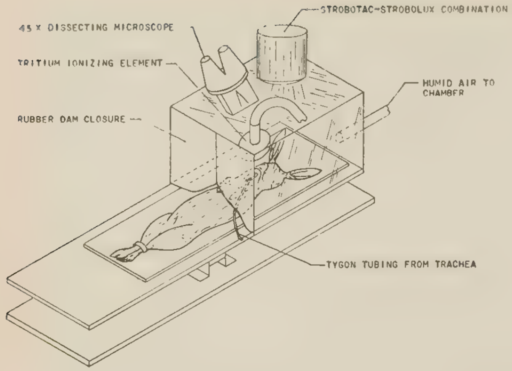
STROBOTAC-STROBOLUX COMBINATION
45 X DISSECTING MICROSCOPE
TRITIUM IONIZING ELEMENT
HUMID AIR TO CHAMBER
RUBBER DAM CLOSURE
TYGON TUBING FROM TRACHEA

FIG. 1. Experimental arrangement for exposing animal trachea to various gases.

with sodium nembutal administered intravenously. The thyroid gland, cricoid cartilage and first 3 to 6 tracheal cartilages were exposed through a midline incision extending from the hyoid bone to the manubrium sterni. So far as possible, adjacent tissues of the larynx and trachea were not disturbed. Next, a longitudinal incision was made into the cricothyroid ligament, care being taken not to penetrate the underlying mucosa. Through this incision the mucous membrane was bluntly dissected from the overlying structures for a distance of one centimeter under the thyroid cartilage cephalad and for 1-2 cm under the cricoid and first 4-6 tracheal cartilages caudad. The exposed cartilages were cut in the midline, the cut ends gently retracted by ligatures, and all bleeders clamped and tied. A perforation barely large enough to admit one blade of a small mosquito forceps was made through the laryngeal mucosa, the blade inserted and the mucosa crushed in the midline for the length of the proposed opening into the trachea. The final incision was made, producing an aperture 1 × 2 cm extending from the lower part of the larynx into the upper part of the trachea. The width could be adjusted by gentle traction on the ligatures tied to the severed ends of the cartileges. To prepare a separate airway, the trachea was intubated as close as possible to the manubrium sterni. The surgical technique was essentially that employed in making the aperture for observation, and as soon as the opening was ready, a snugly fitting polyethylene tube was inserted, connecting the lungs with normal room air (Fig. 1). Two advantages are secured through the laryngeal approach to the trachea; (1) exposure of the large preformed cavity facilitates illumination of the adjacent tracheal field; (2) hemorrhage is minimized and oozing is controlled by the fact that the direction of blood flow is away from the tracheal field and toward the oral cavity. 2. *Humid air chamber.* The head and thorax of the surgically-prepared rabbit was placed inside a glass and plastic chamber so constructed that the mucosa of the trachea could be observed through a microscope during exposure to unionized or ionized gases. To maintain a high humidity the gases were bubbled through water at 60°C before entering the chamber. The temperature within the chamber remained at 25°-26°C. 3. *Gaseous ion source.* Small gaseous ions were generated within the chamber by β radiation from H^3 contained in sealed foils mounted 5-6 cm above the tissue surface. An electrostatic field of low intensity was used to allow selective removal of ions of either charge, leaving an atmosphere containing unipolar ions(4). In some experiments the ground of the rectifying circuit was placed in contact with the trachea just caudal to the viewing aperture, while in others no ground was employed; no difference was noted in the experimental results obtained. Between 0.5 and 1.0×10^9 ions of either charge impinged on each cm^2 of exposed tissue(4).

4. *Determination of ciliary rate.* A strobotactomer (Strobotac-Strobolume combination) was used to determine the rate of ciliary beating as described earlier(1,2).

Results. Observations were made on a total of 8 rabbits breathing normal room air through a separate airway, while the tracheal mucosa was exposed to various gases, ionized and un-ionized. In each case, rate of ciliary beat was determined after a 20 minute period of exposure to a given gas and the vulnerability to trauma was tested by lightly touching the mucosal surface with a blunt probe. Normally this causes no more than a fleeting hyperemia but under some circumstances the vessels may dilate and form a prominent web or a definite ecchymosis appears. Either of the latter conditions is described as enhanced vulnerability to trauma (EVT)(2,5).

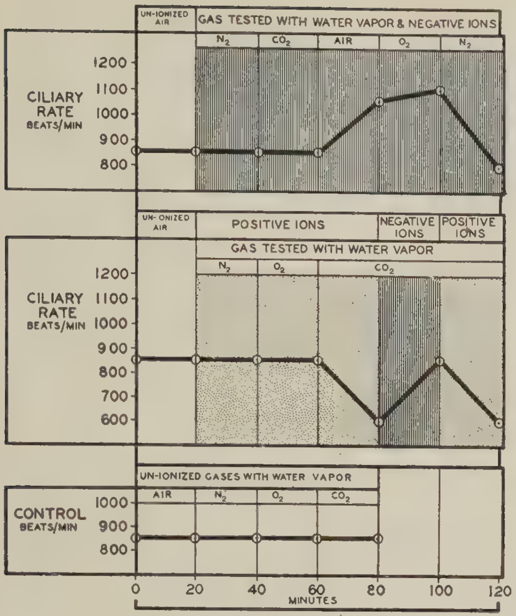

FIG. 2. Effects of ionized and un-ionized gases on ciliary activity in trachea of living rabbit.

Un-ionized N_2, O_2 and CO_2 had no effect on ciliary activity. Under conditions permitting formation of (+)ions, typical inhibition of the ciliary beat occurred in an atmosphere of CO_2 but not in N_2 or O_2. When the rectifying circuit was reversed so that only (–) ions could be generated, atmospheres of N_2 and CO_2 were without effect, while O_2 and ordinary air accelerated the rate of beat (Fig. 2).

N_2, O_2 and air in the un-ionized state or under conditions favoring (–)ion formation failed to induce EVT. This also was true for CO_2 as a rule, but occasionally a barely detectable EVT was produced by untreated CO_2 bled directly from a tank. Probably this resulted from an ionized fraction of the CO_2 produced by natural forces; extremely small numbers of positively charged CO_2 ions will induce a state of EVT(5). With the rectifying circuit reversed so that only (+)ions would be generated N_2 and O_2 were without effect, but CO_2 or air containing CO_2 permitted the prompt formation of a marked EVT.

These findings corroborate and extend our earlier experiments conducted with excised tracheal strips and living tracheotomized rabbits.

It is clear that positively charged CO_2 is the agent responsible not only for reduction of ciliary activity but also for the following effects: (1) Contracture of the trachael smooth muscle; (2) Pronounced mucosal ischemia; (3) Exaggerated lability of the mucosal vessels (enhanced vulnerability to trauma).

Summary. The trachea of the living rabbit was observed during exposure to un-ionized and ionized N_2, O_2 and CO_2 using a separate airway for respiration. Positively charged CO_2 was found to be responsible for: reduction of ciliary activity, contracture of smooth muscle, ischemia and enhanced vulnerability to trauma.

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Received July 28, 1959. P.S.E.B.M., 1959, v102.

Emergence of Hepatitis Virus in Mice Injected with Ascites Tumor. (25247)

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An ascites tumor that originally arose in Princeton mice during transfer of *Eperythrozoon coccoides* was reported from this laboratory in 1956(1). After a long series of passages with ascitic fluid, carrying the tumor cells, lesions that were similar to those produced by the virus of murine hepatitis, MHV (Pr) (2), were observed in the livers of the injected mice. The hepatic reaction was reproducible in the absence of the neoplastic cells and was indeed referable to a virus of the mouse hepatitis group.

Material and methods. The ascites tumor, which closely resembled the Ehrlich tumor, had been maintained for several years by weekly transfer in Princeton mice. Weanlings, 4 to 5 weeks old, were injected intraperitoneally in groups of 5 with .1 ml of undiluted fluid or of a 10% saline dilution. They were killed and autopsied on the 7th day. At this time, they usually showed marked abdominal distension and yielded a considerable amount of creamy peritoneal fluid, either white or slightly tinged with pink. Tumor cells were demonstrable by phase microscopy. Extension of the holding period was followed by massive peritoneal hemorrhage which resulted in a blood-red fluid and death by the 10th to the 14th day. The visceral organs were not involved and the livers were normal in appearance. Swiss mice were equally susceptible but commonly showed terminal hemorrhages with a deep red fluid by the 7th day after injection.

Results. Ascitic fluid was passaged 131 times in 655 Princeton weanlings with no significant departure from the described course of events. In the 132nd passage, however, 2 of 5 mice showed diffuse necrosis of the liver and 3 discrete surface foci. Free fluid was present in the peritoneal cavity but was altered in amount and color. Similar results were obtained with 2 additional passages and it was obvious that a contaminating agent had appeared. The nature and distribution of the liver lesions were pathognomonic of mouse hepatitis. Cell-free liver suspensions were active on injection and were usually free from bacteria. The associated agent was filterable through Coors filters (No. 3) and was identified as a virus of the murine hepatitis group.

Attempts to eliminate the virus by successive washings of the tumor cells with saline solution were unsuccessful. Swiss weanlings known to be less responsive than those of the Princeton strain were used in a second attempt to eliminate the contaminant. This method was also unsuccesful but injections with undiluted ascitic fluid were continued for 10 passages in both mouse strains. Continued development of the virus was indicated by alteration of the peritoneal fluid and involvement of the liver. Both reactions were much more conspicuous in Princeton than in Swiss mice.

Additional experiments were carried out to determine the behavior of the virus in the absence of the tumor cells. An intraperitoneal passage series was begun in Princeton weanlings with a washed liver suspension from 3 of the mice in which hepatic lesions were first observed and continued for 20 transfers. Liver suspensions from the 6th through the 14th passage were also injected intraperitoneally in Swiss weanlings. In addition, 3 successive liver transfers were made in Swiss mice beginning with the 17th passage. The 3rd suspension from the latter series was tested for activity by injection in Princeton weanlings.

The results of the passage series in Princeton mice are summarized in Table I. By reason of an increase in virulence of the virus on continued injection, the series is divided into 2 groups of 50 mice each. The survivors were killed and autopsied on the 7th day. With the exception of one mouse which showed paralysis of the hind legs, the pathologic reaction was limited to the liver and characteristic of hepatitis virus.

All of the 60 Swiss mice survived and were normal in appearance when brought to au-

TABLE I. Intraperitoneal Injection of the Virus Alone in Princeton Mice.

				Liver reaction in survivors		
				No. with		
No. of passages	No. of mice	No. of deaths	No. of survivors	Discrete lesions	Confluent lesions	No lesions
1-10	50	2	48	35	6	7
11-20	50	15	35	15	20	0
Totals	100	17	83	50	26	7

topsy on the 7th day. Three showed a few scattered hepatic foci. The livers from the other mice were normal in the gross and on examination with the dissecting microscope. In the absence of hepatic lesions, active virus was demonstrable in the livers of the mice that received the 3rd passage suspension. Subperitoneal injection in susceptible Princeton weanlings was attended by a characteristic liver reaction.

The ascites tumor had been carried meanwhile, in an uncontaminated state, by successive intramuscular injections in Princeton mice. A second intraperitoneal series was begun with a suspension of the resulting solid growths from several of these mice and has now been maintained for 95 passages in the absence of the virus. Ascitic fluid from this series was used to determine the behavior of an artificial mixture of the uncontaminated tumor cells and the virus. A stored liver suspension from the 12th transfer of the contaminated fluid in Princeton mice was the source of the virus. Fourteen subsequent passages were made with undiluted ascitic fluid at weekly intervals.

The results of this series were similar to those in Princeton mice injected with the naturally contaminated fluid. Changes in the physical state of the ascitic fluid were regularly accompanied by liver lesions.

Pathologic findings. The color change in the contaminated fluids varied from a barely detectable yellowish tinge to a distinct lemon yellow and was presumably due to the influx of bile salts. There were no outward indications of jaundice. Tumor cells were regularly demonstrable in the fluids regardless of their physical state. In the more viscous ones the cells were closely packed and sometimes aggregated. In the limpid fluids they were markedly reduced in number and commonly discrete. At a magnification of 950 diameters with the phase microscope many of the cells showed degenerative changes but normal ones were always present. The fluids also contained cellular debris and spherical masses, with no internal structure, which appeared to be pinched off from the cytoplasmic rim of the cells.

The liver reactions produced by the contaminant alone and by MHV(Pr) were essentially similar but differed somewhat in detail. In both cases, the prodominant histologic feature was necrosis, either diffuse or focal, with little or no cellular infiltration. On examination with the dissecting microscope the livers from mice infected with the present virus showed many, pale, opaque areas separated by narrow red strands or smaller discrete ones with red centers. This admixture of color was not characteristic of the hepatic reaction produced by MHV(Pr). In the livers of some mice, particularly Swiss, frank petechial hemorrhages were also present.

Neoplastic cells were rarely observed in liver sections from mice injected intraperitoneally with the tumor cells alone. In the presence of the virus, however, nests of tumor cells, varying in size and number, were often present and replaced the normal liver parenchyma. Not infrequently, the larger of these cell groupings showed a central area of necrosis.

Discussion. Despite the difference in virulence, it seemed appropriate, at least tentatively, to regard the present virus as a variant of MHV(Pr). On its emergence and subsequent passage the characteristics of the neoplastic cells and the ascitic fluid were noticeably altered. Retrogressive changes were more pronounced in Princeton mice than in Swiss. The virus was readily maintained in both mouse strains by intraperitoneal injection of the contaminated ascitic fluid. The infected tumor cells showed varying degrees of degeneration which resulted in a suppression of growth but not in complete inhibition.

The behavior of the 2 agents in the mouse was suggestive of a synergistic reaction. The virus, in some unknown way, provided favorable conditions for migration of the neoplastic cells to the liver. The tumor cells, in turn, constituted a suitable milieu for multiplication of the virus and also acted as a vehicle for its carriage. In the highly susceptible Princeton mouse there was little indication that the pathogenicity of the virus was appreciably affected by the tumor. Liver lesions occurred as readily in its absence as in its presence. In the more resistant Swiss mouse, however, activity of the virus was increased and the liver reaction significantly enhanced. There was reason to believe that the attendant necrosis began in the nests of tumor cells and spread outwardly to the adjoining parenchyma.

It is probable that the virus was actually carried by one or more mice of the ascites passage series before it was detected by the appearance of liver lesions. The ascites tumor may now be added to the list of agents which have been instrumental in activation of viruses of the murine hepatitis group. In addition to the tumor, the list now includes *Eperythrozoon coccoides,* Niven *et al.*(3), leukemic lymphocytes, Nelson(2), and the reagents urethane and methylformamide, Braunsteiner and Friend(4). The initial emergence of hepatitis virus in Princeton mice has never been observed in the absence of one or another of these agents.

Summary. 1) A contaminant, subsequently identified as a virus of the mouse hepatitis group, appeared in Princeton weanlings during the 132nd intraperitoneal passage of an ascites tumor. The virus was regularly demonstrable in the ascitic fluid and was readily maintained in both Princeton and Swiss mice by serial transfer of fluid. In its presence, livers showed necrotic lesions and the ascitic fluid was altered in amount, consistency, and color. An artificial mixture of the 2 agents behaved in much the same way as the natural mixture. 2) The 2 mouse strains were equally susceptible to the tumor but not to the virus. Liver lesions produced by the virus alone were much more pronounced in Princeton mice. Presence of both agents facilitated migration of tumor cells to the liver and resulted in a marked enhancement of the hepatic reaction in Swiss mice.

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Received July 28, 1959. P.S.E.B.M., 1959, v102

Delayed Development of Righting Reflexes in Offspring of Manganese-Deficient Rats.* (25248)

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Our previous reports(1,2) have shown that a maternal dietary deficiency of manganese in rats and guinea pigs results in the birth of offspring which exhibit ataxia with incoordination and loss of equilibrium. These symptoms appear to result from a congenital defect or defects occurring during latter part of gestation in the rat(1). Observation of defective young during early part of life span raised questions concerning development of the righting reflexes. The present communication reports results of tests designed to examine the effect of a maternal manganese deficiency in rats on development of righting reflexes in their young. Two tests were used: (1) time the animal required to right itself when placed on its back, and (2) ability of animal to right itself in air when dropped upside down.

Methods. Procedures used to obtain manganese-deficient offspring were the same as previously reported(1). Briefly, female rats of the Sprague-Dawley strain were maintained from time of weaning on fortified milk diet deficient in manganese. When they reached maturity, they were mated with normal males receiving a stock diet, and the resulting offspring were tested. First, second and third litters were used. Control animals were treated in the same way, except that the diet was supplemented with manganese. Turnover time was tested beginning at 1 day of

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* This investigation supported in part by research grant from Nat. Inst. of Arthritis and Metab. Dis., P.H.S.

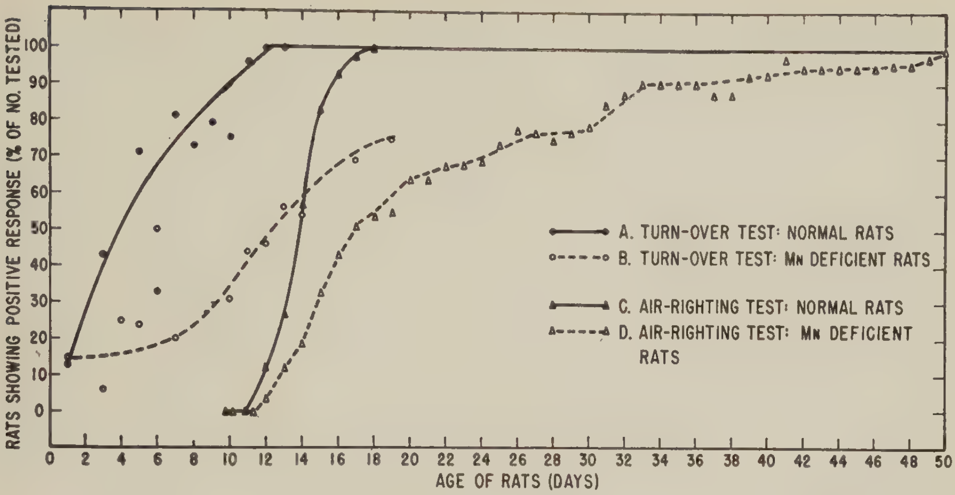

FIG. 1. Development of righting reflexes in offspring of normal and manganese-deficient rats. Curves A and B represent percentage of animals able to turn over in 2.5 sec. or less when placed on their backs. Each point represents tests on from 7 to 34 animals. Curves C and D represent percentage of animals showing a positive air-righting response. Number of normal animals tested was 162. Number of deficient animals tested ranged from 89 at 10 days of age to 42 at 50 days of age.

age. The animals were considered 1 day old 24 hours after birth. The animal was placed back downwards on a table, and the time required to turn over to its feet was measured with a stop-watch. Appearance of air-righting reflexes was tested in young beginning at 10 days of age. Each animal was held back downwards, and dropped 12 inches onto a soft cotton-filled pad. The response was recorded as positive if the rat landed on all 4 feet in 2 out of 3 trials.

Results. The results are summarized in Fig. 1. For the turn-over test (Curves A and B), the response was considered positive when the animal was able to turn from its back to its feet in 2.5 seconds or less. In both normal and deficient groups, this response developed at an earlier age than did the air-righting reaction, but a delay in appearance of the response was exhibited by the deficient animals. At 12 days of age, 100% of normal animals were capable of turning over in 2.5 seconds or less, while at the same age only 48% of deficient rats were able to do so. In addition, examination of individual data showed that until 13 days of age, many of the deficient animals required more than 60 seconds to right themselves, while after 3 days of age, not one of the normal animals needed more than 16 seconds to turn over.

The data concerning air-righting reaction (Curves C & D) also show a significant difference between response of manganese-deficient young as compared with normal young. At 18 days of age, 100% of 162 normal animals were able to right themselves in air, while at the same time, only 54% of 52 manganese-deficient animals showed a positive response. Not until 50 days of age did 100% of the 42 deficient rats tested exhibit the positive reaction. Since only 42 of the original 89 deficient animals tested at 10 days of age survived to 50 days, the data do not, of necessity, include throughout the age span those animals most severely affected by the deficiency. The data may therefore be biased in the direction of a smaller difference between the 2 groups than was actually true. It was not possible to eliminate the data obtained from animals which did not survive, because individual young in litters were not marked.

Discussion. The nature of the defect responsible for the congenital ataxia of manganese-deficient offspring has not as yet been ascertained. No histological abnormalities have been found in the nervous system(1,3). The present experiments indicate at least that the nervous system is involved in the ataxic symptoms observed in manganese-deficient offspring.

That the reflexes concerned with the turnover test developed earlier than did those involved in the air-righting response is in accord with the work of Windle and Fish(4), who reported that body-on-body righting reflexes appeared in the fetal cat before labyrinthine reflexes could be observed.

The delayed development of the air-righting response in manganese-deficient offspring may be related to (a) impaired labyrinthine function(5,6,7) (although Hill *et al.*(8) found no differences between normal and manganese-deficient rats in histological studies of the inner ear), and (b) delayed or abnormal myelination. Windle *et al.*(9) observed some correlation between development of labyrinthine reflexes and myelination in the fetal cat. Furthermore, in the normal animals studied in the present experiments, the air-righting response appeared at the age at which myelination is being accomplished(10). The work of Eayrs and Taylor(11) indicates that the thyroid also may be involved, since thyroidectomized animals showed some delay in development of this response. These possibilities are currently under investigation.

Summary. Development of righting reflexes was studied in offspring of manganese-deficient and normal rats by 2 tests: (1) time required to turn from their backs to their feet and (2) ability to right themselves in air when dropped upside down. The reflexes concerned with the turn-over test developed at an earlier age than did those involved in the air-righting response. In both tests, however, the manganese-deficient young exhibited a marked delay in development of reflexes responsible for righting reactions.

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Received July 28, 1959. P.S.E.B.M., 1959, v102.

Effect of Vit. E-Deficiency on Protein Composition of Guinea Pig Skeletal Muscle.* (25249)

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Several reports concerning the status of one or more muscle protein fractions from animals maintained on a vit. E-deficient diet have appeared in the literature. Spencer *et al.*(1) observed an increase in collagen content of nutritionally dystrophic rabbit muscle. The myosin and actomyosin content of rabbit skeletal muscle fibers gradually decreases with increasing duration of the deficiency state(2). Baechtel *et al.*(3) noted that nitrogen concentration of water soluble extracts of muscles from supplemented and deficient rabbits were the same. In 21-day old vit. E-deficient rats, Rumery *et al.*(4) found a pronounced loss in actomyosin associated with a marked elevation of insoluble protein. The present investigation was initiated to examine the effect of vit. E-deficiency on the sarcoplasmic, contractile and collagen fractions of skeletal muscles from young guinea pigs. It was also of inter-

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* Aided by grant from Muscular Dystrophy Assn.

† Submitted in partial fulfillment of requirements for M. Sc. degree, State Univ. of Iowa, Iowa City.

TABLE I. Means and Standard Errors of Total Nitrogen, Sarcoplasmic Nitrogen, Contractile Nitrogen, and Collagen Nitrogen of Gastrocnemius and Masseter Muscles of Guinea Pigs.

Regimen and duration		No. of cases	Total N	mg nitrogen/g fresh muscle Sarcoplasmic N	Contractile N	Collagen N
				Gastrocnemius		
15 days	Def.	7	31.18 ± 1.51	7.70 ± .62 (24.7 ± .78)	16.88 ± 1.02 (54.2 ± 1.55)	6.60 ± .21 (21.1 ± 1.89)
"	Suppl.	7	31.56 ± 1.91	8.02 ± .67 (25.4 ± .87)	17.25 ± 1.07 (54.6 ± .95)	6.30 ± .11 (20.2 ± 1.30)
21 days	Def.	13	31.06 ± .65	8.32 ± .34 (26.8 ± .70)	15.05 ± .56 * (48.5 ± .89)	7.69 ± .32 * (24.8 ± .81)
"	Suppl.	10	32.34 ± .93	8.22 ± .28 (25.4 ± .89)	18.07 ± .66 (55.9 ± 1.01)	6.05 ± .35 (18.7 ± .76)
"	Recovery	10	33.17 ± .64	8.54 ± .22 (25.7 ± .65)	17.56 ± .46 (52.9 ± .81)	7.07 ± .23 (21.3 ± .33)
30 days	Def.	7	31.72 ± .45	8.73 ± .47 (27.4 ± 1.13)	14.87 ± .18 * (47.0 ± .93)	7.97 ± .10 * (25.2 ± .50)
"	Suppl.	6	31.88 ± .33	8.31 ± .23 (26.7 ± 1.18)	17.00 ± .78 (53.3 ± .70)	6.56 ± .28 (20.5 ± .76)
				Masseter		
15 days	Def.	7	30.81 1.61	8.65 ± .80 (28.1 ± 1.15)	16.00 ± .91 (52.0 ± 1.55)	6.16 ± .71 (20.0 ± .63)
"	Suppl.	7	31.59 ± 1.82	8.39 ± .37 (26.5 ± 1.90)	17.26 ± 1.33 (54.8 ± .74)	5.94 ± .47 (18.8 ± 1.57)
21 days	Def.	13	32.00 ± .61	8.32 ± .23 (26.1 ± .60)	15.99 ± .46 (50.0 ± .86)	7.69 ± .22 * (24.0 ± .61)
"	Suppl.	10	32.85 ± .87	8.68 ± .26 (26.4 ± .57)	18.05 ± .88 (55.0 ± .31)	6.11 ± .35 (18.6 ± 1.24)
"	Recovery	10	32.60 ± .59	8.49 ± .22 (26.0 ± .81)	16.94 ± .51 (52.0 ± 1.01)	7.17 ± .26 (22.0 ± .42)
30 days	Def.	7	30.84 ± .46	8.22 ± .42 (26.6 ± 1.34)	15.79 ± .28 (51.2 ± 1.05)	6.85 ± .47 (22.1 ± 1.53)
"	Suppl.	6	31.44 ± .64	8.59 ± .47 (27.3 ± 1.50)	16.56 ± 1.71 (52.8 ± 1.03)	6.29 ± .51 (19.9 ± 1.60)

Values in parentheses indicate percent of total nitrogen. * Probability ≤.01.

est to study the effect on both red (masseter) and pale (gastrocnemius) muscle, for it has been reported(5) that the masseter muscle is the most resistant to nutritionally induced dystrophy.

Methods. Muscle samples employed in this study were obtained from 30-day old male guinea pigs placed on vit. E-deficient and vit. E-supplemented diets for periods of 15, 21, and 30 days. Details of the dietary regimen have been described(6). The method of chemical dissection proposed by Robinson(7) was adapted for use with small samples (15-30 mg fresh muscle), which were removed from central areas of the muscles with the purpose of excluding, to a large measure, extraneous connective tissue. After the fractionation by means of weak and strong salt extracts, nitrogen content was determined by Kjeldahl digestion followed by direct nesslerization. Results of the nitrogen analyses were expressed as concentration (mg/g fresh muscle) and as percentage of total nitrogen; i.e., the sum of sarcoplasmic, contractile, and collagen nitrogen. A statistically significant difference was accepted only when nitrogen concentration as well as percentage of total nitrogen exhibited a probability of ≤ 0.01.

Results. Table I summarizes the results of nitrogen analyses. When compared to their supplemented controls the experimental gastrocnemius muscles in the 21-day study show, in terms of total nitrogen, a decrease in contractile fraction and an increase in collagen fraction of 7.4% and 6.1% respectively. After 30 days on the dietary regimen, the corresponding changes are: contractile −6.3%, collagen + 4.7%. These differences are significant. In contrast, the masseter data reveal a significant alteration that is limited to the 21-day collagen fraction (+ 5.4%). The sarcoplasmic fraction of either muscle exhibited no significant changes at 21 or 30 days. Supplementation after 14 days on the deficiency diet (both control and recovery animals received 15 mg dl-alpha-tocopherol daily from 15th through 20th days) stabilizes the collagen content of both muscles at or close to 15 day deficient levels; and produces a slight, though not significant, increase in the contractile component of the gastrocnemii. The contractile fraction of recovery masseters is equal to that found for 15-day deficient masseters.

Discussion. Values for nitrogen fractions of control muscles expressed as % of total nitrogen agree closely with those obtained by Hasselbach and Schneider(8) using a somewhat similar extraction procedure on normal rabbit skeletal muscle. With increasing durations of the deficiency state in guinea pigs, there are significant alterations in the contractile and collagen components of the gastrocnemius muscles. On the other hand, with the exception of the 21-day collagen data, masseter muscles reveal no significant change attributable to lack of vit. E. Such results were anticipated by the histological studies of Goettsch and Pappenheimer(5).

According to Spencer *et al.*(1) the gastrocnemius collagen nitrogen concentration of rabbits placed on a vit. E-deficient diet at approximately 30 days of age increased slightly after 4 weeks and markedly after 5 weeks (2x). These authors remark that collagen concentration may be augmented even before outward signs of dystrophy become apparent. In young guinea pigs used for the present study there is a significant change in collagen prior to such evidences of dystrophy as elevated urinary creatine, gross cytological lesions, or decrease in muscle weight.

It is the opinion of Bonetti *et al.*(2) that the decline in actomyosin and complete loss of myosin from dystrophic rabbit skeletal muscle is not merely due to a diminished extractability, but to a real loss of such proteins. For comparison it should be noted that their animals were sacrificed after a sharp decline in body weight had occurred.

Total nitrogen extractable with distilled water from skeletal muscle of rabbits showing typical signs of dystrophy is the same as from control animals(3). The results of the present study on guinea pigs tend to confirm the above findings, but distinctly precede them in terms of severity of the dystrophic state.

Finally, it is of interest to note that the alterations in contractile and collagen protein which occur are matched concurrently by changes in myoglobin concentration(6).

Summary. The gastrocnemius muscles of 30-day-old guinea pigs subjected to a vit. E-deficient diet for 21 or 30 days undergo a statistically significant increase in collagen nitrogen and decrease in contractile protein nitrogen. Masseter muscles are more resistant to the effects of vit. E-lack on the protein components. Sarcoplasmic nitrogen is not altered by 21 or 30 day dietary deficiency regimens.

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Received July 28, 1959 P.S.E.B.M., 1959, v102.

Short Term Studies of Effect of Heparin upon Cholesterol Excretion in Man.* (25250)

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Ample evidence exists that plasma cholesterol is influenced by the amount of circulating triglyceride, and that cholesterol functions as a component of lipoproteins involved in transport of neutral fat(1,2). Chylomicra and lipoproteins bind cholesterol(3-6). It therefore was interesting to investigate the effect of a marked reduction in serum triglycerides upon serum cholesterol, and upon excretion of cholesterol end-products. This could be accomplished by injection of adequate doses of heparin resulting in production of a lipolytic enzyme, lipoprotein lipase, which markedly enhances removal of neutral fat from the blood but has no known direct effect upon cholesterol *per se*. Fecal bile acids and digitonin-precipitable sterols were determined as both pathways apparently function in elimination of cholesterol in man(1,2,7-10).

Methods. Four males were used in this study. Three were normal individuals and one (Z.T.) was a patient with hypercholesterolemia who had sustained a myocardial infarction several years before. The subjects remained on their normal diets during the experiment. All stools were collected over a 4-day period and for 4-6 days during administration of heparin. The latter was injected subcutaneously as a concentrated aqueous solution† in doses of 150-200 mg every 12 hours depending upon the patient's weight. This amount of heparin is the usual anticoagulant dose and affords substantial triglyceride removal from the bloodstream(11). Postabsorptive blood samples were drawn prior to and at the end of the heparin period, and serum cholesterol(12) and triglycerides(13) determined. Feces were analyzed in duplicate for bile acids(14) and digitonin-precipitable sterols(15). The 4-day control sample was pooled, whereas the stools collected while on heparin were grouped as 2 or 4 day pools.

Results. Average values obtained from the duplicate fecal analyses are shown in Table I. An increase in bile acid and digitonin-precipitable sterol excretion occurred during the period of heparin administration in all 4 individuals. Increase in bile acids varied from 25-96%, in sterols from 19-174%. The decrease in cholesterol end-product excretion found in the last 2 days in subject M.S. is probably misleading. It can be seen from the marked reduction in the stool dry weight that he was constipated during this period. Unfortunately the heparin had been discontinued before this was appreciated. The data presented in Table II illustrates the reduction in circulating triglyceride which occurs following heparin therapy, and the smaller decrease in the serum cholesterol. As had been repeatedly noted with other modalities, when the initial cholesterol level is higher a larger drop occurs (patient Z.T.).

Discussion. During these experiments the possibility was suggested that injected heparin appeared in the stool in increased amounts, and that it was not entirely removed by the

TABLE I. Daily Fecal Bile Acids, Digitonin-Precipitable Sterols and Dry Weight before and during Heparin Administration.

Subject	Analysis	Before heparin	Days on heparin* 2	4	6
H.E.	Bile acids†	220	378	326	268
	Sterols	390	1070	693	775
	Dry wt	82	88	66	69
Z.T.	Bile acids	296		580	
	Sterols	276		352	
	Dry wt	78		96	
E.S.	Bile acids	221		277	
	Sterols	308		368	
	Dry wt	91		87	
M.S.	Bile acids	456	659	143	
	Sterols	662	1440	319	
	Dry wt	109	70	26	

* Before heparin sample analyzed as a 4-day pool. After heparin stool samples analyzed as 2- or 4-day pools.

† Values of bile acids and digitonin-precipitable sterols in mg, dry wt in g.

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* Supported by research grants from N.H.I., N.I.H., P.H.S.

† Lipohepin. Supplied through courtesy of Darwin Labs, Los Angeles, Calif.

TABLE II. Fasting Serum Triglycerides and Cholesterol before and after Heparin. Values in mg %.

Subject	Triglycerides Before	Triglycerides After	Cholesterol Before	Cholesterol After
H.E.	144	53	220	206
Z.T.	131	103	420	336
E.S.	157	69	253	235
M.S.	146	73	237	204

bile acid extraction procedure. In view of its acidic nature heparin, if present, could affect the bile acid titration. However we were unable to demonstrate any metachromatic or anticoagulant activity in the final extract after dialysis. Furthermore, heparin added *in vitro* to a stool sample did not appear in the ethanolic extract which contains the bile acids and lipid substances. It therefore appears fairly conclusive that the increased acidity demonstrated in the bile acid extraction procedure after heparin injection was not due to the presence of heparin itself.

There is no known direct effect of heparin or lipoprotein lipase upon cholesterol absorption, synthesis or excretion. It is most likely that the enhanced elimination of cholesterol end-products demonstrated in these subjects after heparin therapy occurred as a result of the decrease in circulating triglycerides. When less neutral fat is present in the bloodstream, less cholesterol is needed for this transport function and the excess is excreted, both as bile acids and sterols. It may also be that some of the increase in digitonin-precipitable sterols resulted from decreased cholesterol absorption secondary to lower serum triglycerides although we have no evidence to support this possibility. In any event the findings of this study confirm that serum triglyceride levels affect cholesterol levels although they are undoubtedly not the only factor involved in the genesis of hypercholesterolemia. Heparin, by reducing the neutral fat content of the blood(16-17), lowers serum cholesterol in those cases where hypercholesterolemia is secondary to hypertriglyceridemia. Since the turnover rate of plasma cholesterol is much slower than that of fatty acids(18), it is possible that longer periods of heparinization would result in more profound drops in cholesterol than were seen in these short-term experiments.

The data indicate that when loss of cholesterol from the body is increased by any modality it cannot be assumed that this has occurred via a primary effect upon hepatic or intestinal excretory mechanisms. Thus this explanation of the decrease in serum cholesterol observed when vegetable oils are substituted for animal fats may not be valid(9,10, 19-21). Fecal losses of sterols are more rapidly affected than serum changes(21) but this is probably due to the slow turnover time of plasma cholesterol as compared to the hepatic conversion of sterol to cholic acids, and does not indicate that excretion of greater amounts of cholesterol end-products is the cause of reduction in serum cholesterol. It is more logical that cholesterol is lost from the body in larger quantities than usual whenever there is a decrease in the requirements for it, in any of its functions throughout the organism. The effects observed after injection of heparin and lowering of serum triglycerides support this point of view. It is possible that the differential effects of saturated and unsaturated fats partially are mediated through similar mechanisms(22).

The present investigation should be extended to involve more subjects, on controlled diets, and with longer observation periods on heparin therapy. It would also be advisable to study individuals with hypercholesterolemia who do and do not have an associated elevation of serum triglycerides, to obtain further information on the interrelationship of these 2 lipid parameters. However, since the fecal analyses are laborious, it was desirable to present the results of this preliminary study.

Summary. 1. Serum triglycerides and cholesterol were decreased, and fecal bile acids and digitonin precipitable sterols were increased, after injection of heparin in 4 human subjects. 2. Evidence was discussed which indicates that this effect of heparin upon cholesterol metabolism is a secondary phenomenon resulting from maintained reduction of circulating triglycerides.

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Received July 28, 1959. P.S.E.B.M., 1959, v102.

Analysis of Weight and Height of Apparently Healthy Population, Ages 65 to 94 Years. (25251)

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This paper presents a study of average weights at each inch of height for men and women in successive 5 year age groups from 65 to 94 years of age. A review of the literature has failed to reveal a comparable study and we believe that this constitutes the first comprehensive analysis of height and weight in old age. This study represents one facet of a larger survey of blood pressure and health of this old age white population of the United States. Previous publications have dealt with relationship of blood pressure to age and sex (1) of blood pressure to weight and height (2), etc.

Materials and methods. Detailed information concerning collection and statistical evaluation of the data has been presented (1-3). 2925 males and 2694 females were finally accepted for study. Statistical evidence that the sample was reasonably homogenous and representative of aged, clinically healthy, white population of U. S. has been presented(3). Average weight at each inch of height was computed separately for each sex and 5 year age group. A more consistent progression of weight with increasing height was then obtained from these crude data by use of weighted moving average, a commonly employed statistical procedure for this type of data.* The derived figures were plotted and freehand curves fitted to them. No attempt was made to achieve absolute uniformity in profile of these curves.

Results. Table I presents the crude data and number of observations for each inch of height. Height range in males was essentially from 61″ to 73″; while in females from 58″ to 68″. Females consistently weighed less than

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* Weight in lbs for each inch of height represents a weighted average of values for 5 consecutive inches from 2 below to 2 above inclusive. Weighting values of 1, 4, 6, 4, 1, were assigned. Average weights based on fewer than 5 frequencies were omitted. With this technique, weight obtained for every inch of height is actually based upon roughly 5 times as many observations as in the crude data and "weighting" tends to reduce distortion occurring at extremes of height due to unequal frequencies.

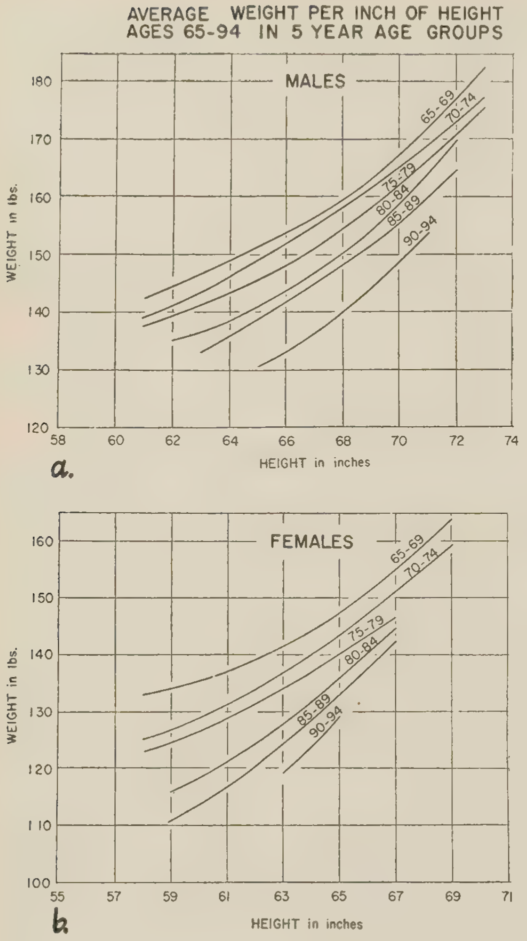

FIG. 1.

males of same height. Weight in both sexes increases in curvilinear fashion with increasing height.

Fig. 1A and 1B are presentations of the weight per inch of height derived from Table I by use of a weighted moving average. Thus, in both sexes at every inch of height, there is a decrease in average weight of progressively older 5 year age groups. Average decrease in weight with advancing age seems to be similar in both sexes. However, while among males decrease in weight with age is uniform throughout all levels of height, among women, those of short height experience a greater decrease than tall subjects. Therefore, it would seem that short individuals of both sexes, but primarily females, lose a greater proportion of their body weight than tall persons with adding age after 65, though this may simply represent weight changes of different surviving generations.

A study of modal and mean heights of 5 year age groups of both sexes and standard deviation of frequency distribution of height was made. It is significant that neither the mean nor modal height show any consistent decrease with advancing age. This indicates that the average height of surviving populations remains virtually unaltered after 65 years, being about 67″ in males and 62″ in females. The standard deviation for height remains constant after 65 years. Therefore, approximately 67% of male subjects are between 64″ and 70″ tall, and approximately 67% of females between 59″ and 65″. The fact that both mean height and standard deviation remain constant after age 65 years indicates that the distribution of height has not changed materially. This suggests that these subjects have neither lost height nor has mortality or morbidity favored any particular height group as compared with any other.

Discussion. Decrease in weight with age after 65 probably can be explained as the additive result of 2 factors: 1, loss of body substance, *i.e.*, protein, water, fat and calcium which is the physiological accompaniment of aging(4,5); 2, progressive reduction in proportion of overweight subjects due to relatively higher mortality rate among obese as compared with "underweights."

From our analysis of the mean, modal height and standard deviation of height in each 5 year age group, it appears that survival is not affected by height in this older age group. This is somewhat contrary to previous investigators(6) who reported that up to age 40 tall persons had a somewhat poorer survival rate than those of short or average height.

Limits of desirable weight variation of approximately ± 20% are traditional with insurance companies, but we feel these a bit broad and that limits of ± 10% are more suitable.

Summary. 1) Average weights per inch of height for both sexes in older population, ages 65 to 94, have been presented. This is the first study of its kind to appear. It is based

TABLE I. Weight per Inch of Height—Ages 65-106 in 5 Year Age Groups. Crude data.

Height	65-69		70-74		75-79		80-84		85-89		90-94		95-106	
(inches)	N	Wt	N	Wt	N	Wt	N	Wt	N	Wt	N	Wt	N	Wt
					Males (total 2925)									
52					1	160								
53														
54			1	138									1	125
55														
56							1	124						
57			1	157					1	105				
58			2	131					2	122				
59	2	122	3	130	1	132	5	140	1	90				
60	10	149	6	149	4	125	2	148	6	121	3	130		
61	13	144	7	131	5	152	8	130	2	116	2	118	1	107
62	24	140	20	140	6	146	11	140	8	115	4	126	2	122
63	41	148	29	147	19	148	9	128	12	132	6	144		
64	66	148	39	146	42	142	27	140	27	140	12	128		
65	77	152	51	154	44	148	21	138	24	144	12	130	5	146
66	100	155	89	153	74	145	58	149	37	141	11	132	2	140
67	93	155	93	154	69	155	53	148	26	146	19	135	4	138
68	126	159	89	158	73	154	55	148	36	146	13	139	5	163
69	76	163	81	163	47	154	32	157	29	156	7	148	1	147
70	80	167	61	165	55	161	45	156	31	158	9	147	2	165
71	79	172	45	174	40	158	20	159	28	163	9	154		
72	62	182	31	172	22	173	17	166	24	160	7	163	1	200
73	18	180	20	172	12	182	9	187	10	169	3	154		
74	11	186	6	180	7	188	2	142	3	185	2	159		
75	5	171	4	146					1	170				
76	4	162	3	197										
77							1	125	2	162	1	135		
78							1	165	1	180				
79			1	138										
80									1	148				
81									1	135				
82	1	185												
Total N	888		682		521		377		313		120		24	
					Females (total 2694)									
49	1	108												
50	1	96												
51	1	175	1	110										
52	1	155	2	108										
53											1	139		
54			1	145	2	101	1	87	2	124	2	90		
55	1	120	3	126			3	118	2	90				
56	6	138	6	124	6	118	2	88	5	114				
57	14	135	11	142	6	126	3	121	3	133	2	104		
58	30	133	24	137	7	119	12	116	8	113	4	86	1	110
59	42	132	22	123	17	117	9	106	11	109	4	114		
60	91	135	64	129	53	126	44	122	32	110	22	108	2	100
61	92	138	72	129	61	131	49	123	29	119	12	124	3	109
62	123	139	102	138	70	136	48	125	37	125	19	125	6	122
63	122	139	97	136	71	134	54	129	30	125	12	112	1	107
64	120	143	102	136	52	138	58	133	34	130	12	121	7	136
65	70	150	68	143	42	142	20	126	24	130	12	131	1	118
66	61	156	38	153	33	138	9	153	14	139	10	141	2	124
67	25	154	29	154	10	148	12	136	8	135	4	150	1	147
68	19	158	12	138	11	142	8	155	7	159	2	150		
69	9	169	3	149	3	111	1	190	2	134				
70	8	148	5	174	4	164	2	172	2	130	2	165		
71	1	150	3	154	2	170			2	135				
72	1	150			3	175								
73			2	160										
74							1	214						
75	1	189												
76	1	147	1	148										
Total N	841		668		453		336		252		120		24	

on a population sample of apparently healthy persons and the sample size is considered statistically adequate. 2) There exists a definite trend towards a decrease in average weight of successive 5 year age groups after age of 65. There were 2 reasons for this: 1) individual weight loss accompanying aging due to loss of body substance, 2) high mortality rate among obese which favors survival of a larger proportion of underweight people. 3) Height and survival were not related.

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Received July 30, 1959. P.S.E.B.M., 1959, v102.

New Inhibitors of *in vitro* Conversion of Acetate and Mevalonate to Cholesterol. (25252)

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The *in vitro* conversion of labeled acetate (1) and labeled mevalonate(2) into cholesterol by rat liver homogenates have been reported, as well as the effect of known hypocholesteremic agents upon these syntheses(3,4). The present studies are concerned with the effect of several new compounds upon the enzymatic synthesis by rat liver homogenates of cholesterol from both acetate and mevalonate. The compounds are Δ1-testololactone, Δ4-androstene 17-α-ol-3-one-17β-oic acid, and β-hydroxy - β - fluoromethyl - δ - valerolactone, (fluoromevalonic acid).* The effects of fluoride ions, fluoroacetate, and zinc ions upon these conversions have also been investigated.

Methods. Livers were obtained from 100 g Sprague Dawley rats maintained on Purina Lab Chow. The animals were killed by decapitation and their livers quickly removed and placed on ice. Homogenization was carried out in the manner reported by Bucher(1), in 2.5 volumes of .1 M phosphate buffer of pH 7.0.† Five ml of the supernatant solution after refrigerated centrifugation at 700 x G for 10 minutes were used as the source of enzyme for each determination. To use the identical enzyme preparation for all levels of compounds studied in each test, several livers were pooled and 5 ml aliquots used for each level and for controls. To each aliquot was added .5 mg of DPN and .5 mg of ATP in .1 ml volumes. Twenty-five μg of 2-C^{14} acetate (24.4 μc/mg) contained in 1 mg of K acetate in .1 ml was the acetate substrate. Forty μg of 2-C^{14} mevalonic acid (1.6 μc/mg as the dibenzylethylenediammonium salt) in .1 ml volume was the mevalonate substrate. The mevalonic acid was liberated from its salt by treatment with alkali and extraction with ether(5). Compounds under test were added either as a fine suspension or as a solution in a .2 ml volume of 5% propylene glycol. The enzymatic synthesis was carried out in 50 ml Erlenmeyer flasks placed in a Dubnoff metabolic shaker under an atmosphere of 95% oxygen-5% CO_2. The samples were incubated for 2 hours at 34°C. After this period .5 mg of cholesterol was added to each flask and the entire contents saponified with alcoholic KOH in a

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* The authors are indebted to Drs. J. Fried for supplying steroids and R. Tschesche, of Hamburg, Germany, for supplying flucromevalonic acid.

ml
† 4 1M KH_2PO_4
6 1M K_2HPO_4
5 .1M $MgCl_2$
3 1M Nicotinamide
82 deionized water

TABLE I. Effect of Steroids on Acetate and Mevalonate Conversion to Cholesterol.

Compound	mg	Acetate conversion						Mevalonate conversion			
		Test 1		Test 2		Test 3		Test 1		Test 2	
		cpm/plate	% inhib.	cpm/plate	% inhib.	cpm/plate	% inhib.	cpm/plate	% inhib.	cpm/plate	% inhib.
Δ4-Androstene-17α-ol-3-one-17β-oic acid	2.0	153	97			0	100	5,976	73		
	1.0					3	100	14,836	32		
	.5					427	57				
	.25							24,140	11*		
	.067							21,485	2		
Δ1-Testololactone	2.0			857	92	125	87			26,755	27
	1.0					772	23			34,753	6
	.25									36,422	1
	.067									39,075	6*
Control		4,744		10,274		990		21,979		36,848	

The radioactivity added was 650,000 cpm for acetate and 130,000 cpm for mevalonate. * Values in excess of control are expressed as acceleration.

boiling water bath under a stream of nitrogen. When the alcohol had evaporated, the saponification was considered complete(6). After acidification, the solution was extracted with ether and the cholesterol was isolated as the digitonide from the ether extract. The digitonide was dissolved in 1 ml of absolute alcohol, and evaporated on an aluminum planchet under an infra-red lamp. Counts were made on a gas-flow proportional counter (Tracerlab-Deciscaler), corrected for self-absorption, and expressed as counts/minute/plate. In these studies the radioactive digitonide precipitate was considered as a precipitate of cholesterol only, based on the report of Frantz and Bucher(7) that the digitonin precipitable material produced by cell free homogenates is approximately 97% cholesterol.

The results of several studies where Δ4-androstene-17α-ol-3-one-17β-oic acid and Δ1-testololactone have been added to the liver homogenate system are shown in Table I. Both of these compounds are effective in inhibiting the conversion of acetate to cholesterol. When mevalonic acid was used as the substrate, Δ4-androstene-17α-ol-3-one-17β-oic acid showed marked inhibition at the 2 mg level, while Δ1-testololactone was much less effective at this concentration.

The best inhibition of both systems was obtained with fluoromevalonic acid. The results of this study are summarized in Table II. Fluoromevalonic acid appears to be 5-10 times more effective in inhibiting acetate conversion than mevalonate conversion. A 40% inhibition of the acetate system is obtained with .004 mg of fluoromevalonic acid while a 27% inhibition of the mevalonate system is obtained with .031 mg.

To rule out the possibility of fluoromevalonic acid being metabolized to either fluoroacetate or fluoride ion, both of these substances were tested in the range of concentration that would result if such metabolism did occur. The results are presented in Table III. Neither substance had any effect on either acetate or mevalonate conversions.

It is of interest to note that addition of .5 mg of unlabeled mevalonic acid to the acetate system caused only a 7% reduction in counts while .5 mg of fluoromevalonic acid caused a

TABLE II. Effect of Fluoromevalonic Acid on Acetate and Mevalonate Conversion to Cholesterol.

		Acetate conversion		Mevalonate conversion			
		Test 1		Test 1		Test 2*	
Compound	mg	cpm/plate	% inhib.	cpm/plate	% inhib.	cpm/plate	% inhib.
Fluoromevalonic acid	2.0			881	97		
	1.0	215	95	1,392	96		
	.5					11,720	85
	.25	333	92			11,473	79
	.125					29,030	66
	.063	495	89			39,872	51
	.031					59,526	27
	.015	1,580	66				
	.0039	3,064	40				
	.0009	4,834	3†				
Control		4,764		32,224		81,301	

Radioactivity added was 650,000 cpm for acetate and 130,000 cpm for mevalonate.

* 80 μg of labeled mevalonic acid used in this study with an activity of 260,000 cpm.

† Values in excess of control are expressed as acceleration.

TABLE III. Effect of Fluoride, Sodium Fluoroacetate, and Zinc on Acetate and Mevalonate Conversion to Cholesterol.

		Acetate conversion		Mevalonate conversion	
Compound	mg	cpm/plate	% inhib.	cpm/plate	% inhib.
Fluoride (as NaF)	.25			22,985	0
	.063			22,492	0
Control				22,993	
Sodium fluoroacetate	.1	4,527	2	37,132	1
	.01			34,882	7
	.001			37,942	1*
Control		4,632		37,444	
Zinc (as $ZnCl_2$)	.04	10	99	33,831	9
	.01	382	70	38,101	3*
	.0025	1,368	8*	39,063	5*
Control		1,272		37,045	

Radioactivity added was 650,000 cpm for acetate and 130,000 cpm for mevalonate.

* Values in excess of control are expressed as acceleration.

90% reduction. Addition of 1 mg of unlabeled mevalonic acid gave a 61% reduction in counts in the mevalonate system while 1 mg of fluoromevalonic acid brought about a 97% reduction.

In examining compounds for inhibitory properties, a striking effect of zinc ions was noted. Curran and Clute(8) had found no effect of .0001 M $Zn\ Cl_2$ upon their liver cell cluster conversion of acetate to cholesterol. In our system, a definite inhibition upon acetate conversion was observed while no effect was seen upon mevalonate conversion with .01-.04 mg (.00003M to .00011M) concentrations of zinc ions. These data are also shown in Table III.

Summary. The effects of Δ1-testololactone, Δ4-androstene 17α-ol-3-one-17-β-oic acid, and fluoromevalonic acid upon *in vitro* conversion of labeled acetate and labeled mevalonate into cholesterol were studied. All of these compounds inhibited conversion of acetate, while only the latter 2 inhibited conversion of mevalonate. Fluoromevalonic acid was the most potent inhibitor studied. Fluoroacetate, fluoride ions, and zinc ions had no effect on the mevalonate system. Of these substances only zinc ions inhibited conversion of acetate to cholesterol.

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Received July 30, 1959. P.S.E.B.M., 1959, v102.

Biochemical Changes Occurring in Mouse Liver Cells with Ageing.* (25253)

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A number of workers have investigated biochemical changes occurring in tissues as animals age, but little information is available concerning such changes in the subcellular fractions of tissues. An opportunity arose in this laboratory, in connection with other studies, to measure nitrogen and phosphorus contents of large and small granules and cytoplasmic supernatant in liver cells from mice ranging in age from 1½ to 21 months. The data obtained demonstrate that as animals age changes occur in relative amounts of these constituents in all 3 subcellular fractions examined.

Method. Inbred virgin female mice of the C3H/Sp strain, reared on laboratory chow and water *ad lib* in air conditioned animal rooms, were employed. They were sacrificed by exsanguination via jugular vein and the livers removed without prior perfusion. Large granule, small granule, and cytoplasmic supernatant fractions were obtained by differential centrifugation employing a sucrose medium, essentially as described earlier(1). Nuclear counts were made(2) on tissue suspensions prior to preparation of the cell fractions. Nitrogen(3) and phosphorus(4) were determined on each fraction prepared, and were calculated on a per nucleus basis.

Results are shown in Table I. There was an increase with age in nitrogen content of all cell fractions examined. Increases were seen in the small granule and cytoplasmic supernatant fractions by 6-7 months, and in the large granule fractions by 10-11 months of age. Similar changes were seen in phosphorus contents of these fractions, but they generally were of smaller magnitude. In the oldest animals studied chemical compositions of the fractions had altered, as evidenced by lowered P/N ratios in all 3 fractions. There are probably no significant differences in numbers of binucleate cells present in livers of animals studied regardless of age(5). It therefore seems safe to conclude from the nitrogen data that, as the animal ages, the amounts of large and small granules and cytoplasmic supernatant increase within the cell. This appears to confirm the report of Franzini *et al.*(6) that with age there is a gradual increase of cytoplasm-nucleus ratio in livers of mice.

There were fewer cells/g of liver tissue, as indicated by decreasing numbers of nuclei, with increasing age but this appears to be largely compensated for by an increase in liver mass (Table I). There is probably less mitotic activity in livers of the older than in those of younger animals(5), which might suggest a decreasing vigor of the cells. However, total content of the 3 fractions examined in liver cells increased with age. That this process may be simply a form of compensatory hypertrophy is suggested by the observations of Weinbach and Garbus(7), who reported a lower absolute level of substrate oxidation by liver mitochondria from aged

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* Supported in part by grants from Nat. Cancer Inst., N.I.H., U.S.P.H.S.; and by institutional grants to Detroit Inst. of Cancer Research from Am. Cancer Soc.

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TABLE 1. The Effect of Age on the P and N Contents of Mouse Liver Cell Fractions.

Cell fraction		1½-2 mo	6-7 mo	10-11 mo	18-21 mo
		(12)*	(12)*	(9)*	(7)*
Mitochondria	Nitrogen†	27.7 ± 1.1	29.9 ± 1.4	41.6 ± 1.5	45.4 ± 1.5
	Phosphorus†	3.30 ± .44	3.26 ± .13	4.73 ± .21	4.98 ± .13
	P/N ratio‡	11.9 ± .17	11.0 ± .16	11.4 ± .40	11.0 ± .10
Microsomes	Nitrogen	15.3 ± .6	19.8 ± .6	17.8 ± .7	19.9 ± 1.0
	Phosphorus	2.6 ± .08	3.36 ± .1	2.88 ± .12	3.07 ± .2
	P/N ratio	17.0 ± .3	17.0 ± .3	16.2 ± .2	15.4 ± .3
Supernatant	Nitrogen	34.3 ± 1.1	40.8 ± 1.0	45.9 ± 1.5	47.7 ± 1.6
	Phosphorus	2.95 ± .07	3.62 ± .09	3.82 ± .15	3.42 ± .3
	P/N ratio	8.6 ± .1	8.9 ± .1	8.2 ± .1	7.2 ± .5
Nuclei§		281 ±10.	236 ± 8.7	206 ± 5.7	211 ±11.
Liver wt (g)		1.04 ± .03	1.13 ± .04	1.21 ± .04	1.29 ± .03

* Figures in parentheses are No. of animals employed.

† Values are μg × 10^6/nucleus. Means and stand. errors are given.

‡ Ratios are μg P/μg N × 10^2. Means and stand. errors of means are given.

§ Values are No. of nuclei/g moist tissue × 10^{-6}. Means and stand. errors are shown.

rats, without any evidence of uncoupled phosphorylation. Their data indicate that with mitochondria from old animals, oxidation proceeded at about ⅔ the rate seen with mitochondria from younger animals. It is interesting that in the present experiments with mice, the large granule nitrogen increased almost inversely proportional to this decrease in oxidative capacity, indicating that perhaps the old animal has successfully compensated for the deteriorating metabolic activity of mitochondria by forming more mitochondria within each cell.

Whether replacement mitochondria have the same metabolic potential as those in younger animals, and whether or not such compensating actions result in a maintenance of normal vigor is not indicated by these experiments. The altered P/N ratios observed would tend to raise a question in this regard.

Summary. Nitrogen and phosphorus contents of large and small granules and cytoplasmic supernatant from livers of mice 1½-2, 6-7, 10-11, and 18-21 months of age were determined and expressed on a per nucleus basis. There were increased nitrogen contents, and relatively smaller increases in phosphorus contents in each of these fractions in older animals. The results, viewed in the light of observations of Weinbach and Garbus (7), suggest that as an animal ages, its liver cells lose metabolic capacity, and, also, that an attempt is made to restore at least some aspects of it by forming more of the active sites within the cell. Cell fractions examined in older animals differed chemically from those in younger animals, however, as indicated by decreased P/N ratios.

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Received August 10, 1959. P.S.E.B.M., 1959, v102.

Effect of Fatty Acids on Chick Encephalomalacia.* (25254)

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The intake of easily oxidized unsaturated lipids appears to be important in producing encephalomalacia in Vit. E deficient chicks (1). Our studies(2,3) and those by Dam *et al.*(4) and Machlin *et al.*(5) suggested that the amount of linoleic acid-containing oils ingested was of primary importance in the etiology of this disorder. Addition of coconut oil to a diet containing a low level of corn oil increased the incidence of encephalomalacia in tocopherol-deficient chicks, although high levels of dietary coconut oil alone had no effect (3). Effects of various dietary lipids are reported here, using appearance of symptoms of encephalomalacia and cerebellar hemorrhages as end-point. Evidence was obtained that fatty acids other than linoleic acid modify the appearance of symptoms.

Materials and methods. One-day-old Indian River crossbred cockerels in electrically heated brooders were given the diet described by Bosshardt *et al.*(6) with slight modifications(3). Substitutions of lipids other than at 4% level were made isocalorically with dextrose. The low-tocopherol corn oil was the same as that used in our studies of human tocopherol requirements(7,8), having been stripped of most of the tocopherols by vacuum distillation† and stirred with air at 95°C until a peroxide number (PN) of 50 was reached. Lard was similarly stripped and oxidized to PN of 25. Commercially available linseed, coconut, and cod liver oils, and butter were also aerated just sufficiently to oxidize the tocopherols. The lipids were analyzed for peroxide number, polyunsaturated fatty acids, iodine number, and total, alpha, and gamma tocopherol levels as described elsewhere(3), and in addition, some fatty acid compositions were determined by gas chromatographic methods using an ionization detector(9,10). The saturated fatty acids used had the following purities: lauric acid, 98.6%; myristic acid, 97.6%; palmitic acid, 96%; and stearic acid, 99%. Oleic acid assayed at 76.2%, the chief impurities being palmitoleic acid, 9.8%; palmitic acid, 4.2%; and myristic acid, 3.1%; with only 1.8% linoleic acid. Lipids and water-soluble vitamins were added to the diets daily to minimize oxidation. Vit. A (2500 IU) and D (600 IU) were fed weekly, and d-α-tocopherol acetate supplements were given in drops twice/week. Birds having ataxia, clonic, and tonic convulsions, head twisting, or paralysis after 14 and up to 28 or 30 days were sacrificed and the cerebella examined.

Results. The "diene" contents of diets used (Table II) compare favorably with linoleic acid levels reported by Hilditch(11). Approximately 70% of chicks developed characteristic symptoms with cerebellar lesions when fed a low-tocopherol synthetic diet containing 4% stripped corn oil (Table I). The relationship between level of corn oil in the diet and tocopherol necessary to prevent encephalomalacia has been previously described(3). Soy bean oil, cottonseed oil, safflower oil, and lard were also capable of inducing encephalomalacia in tocopherol-deficient chicks when the diets contained at least 14 g of linoleic acid/kg(3). However, neither olive oil nor linseed oil induced encephalomalacia, even when amounts equivalent to 23 or 18 g of linoleic acid/kg diet, respectively, were fed. Ten % cod liver oil by itself, although furnishing high levels of pentaene and hexaene fatty acids, did not produce cerebellar lesions. No direct relationship between iodine number and incidence of encephalomalacia was noted.

When combined in the diet with safflower oil, linseed oil inhibited appearance of encephalomalacia, while olive oil had an insignificant effect. The effects of combinations of

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* Support of Ill. Mental Health Fund, National Vitamin Foundation, Inc., and U.S.P.H.S. Grant is gratefully acknowledged.

† We are indebted to Distillation Products, Inc., for stripping most tocopherol from corn oil and lard used in this study.

TABLE I. Effects of Various Dietary Lipids on the Incidence of Encephalomalacia.

Dietary lipid (vit. E removed)	PN*	Iodine No.	"Diene," g†	Linoleic acid, g‡	Incidence
		per kg diet			
4% stripped corn oil	58	50	22	12–22	48/70
4% ” lard	28	28	5	4– 5	0/10
15% ” ”	28	92	17	12–20	10/20
20% ” ”	28	128	22	16–26	15/21
15% butter	0	63	2.3	1.5–6.0	0/16
20% coconut oil	1	20	3.6	2.6–4.8	0/10
10% olive oil	60	89	11	4–20	2/37
15% ” ”	60	133	17	6–30	0/19
20% ” ”	60	178	23	8–40	”
10% linseed oil	120	183	12§	9–22	”
15% ” ”	120	274	18§	14–33	0/10
10% cod liver oil	45	160	(~1.6)		0/18
10% oleic acid		89	2.2		0/ 9

* PN reached after heating with aeration sufficiently to destroy virtually all tocopherol.

† Diene content, as determined by alkaline isomerization and UV absorption.

‡ Calculated values from Hilditch(11).

§ Linseed oil also furnished 54 and 81 g of linolenic acid, respectively, to 10% and 15% diets.

natural lipids and various fatty acids with corn oil are given in Table III.

Discussion. Encephalomalacia is induced in tocopherol-deficient chicks when the linoleic acid content of the diet is above a certain minimum(3), but this incidence of encephalomalacia is enhanced, unaffected, or inhibited by various fatty acids. The ability of lauric and myristic acids to increase this incidence of encephalomalacia may be due to their being metabolized first, leaving more linoleic acid in tissues, while palmitic and stearic acids, which are not as well absorbed (12), may be less capable of "sparing" linoleic acid. Ability of some lipids and fatty acids to inhibit occurrence of encephalomalacia in corn oil-fed chicks appeared to be related to unsaturated fatty acids which they provide, other than linoleic. Thus, lard and olive oil both furnish equal amounts of

TABLE II. Effects of Various Lipids in Combinations with Safflower Oil on Incidence of Encephalomalacia.

Dietary lipids (treated to remove tocopherols)	Incidence
3% safflower oil	7/11
Idem + 12% olive oil	3/10
” + 12% linseed oil	0/11
5% safflower oil	6/10
Idem + 12% olive oil	3/11
” + 12% linseed oil	0/11

TABLE III. Effects of Various Lipids and Fatty Acids in Combinations with Stripped Corn Oil upon the Incidence of Encephalomalacia.

Dietary lipids (treated to remove tocopherol)	Incidence
2% corn oil	7/40
4 ”	48/70
2 ” + 8% coconut oil	40/51‡
2 ” + 8 butter	9/34
2 ” + 8 olive oil	9/20
4 ” + 6 ”	14/20
2 ” + 8 linseed oil	0/20
4 ” + 6 ”	6/19
2 ” + 8 cod liver oil	0/20
4 ” + 6 ”	1/20‡
4 ” + 6 oleic acid	0/19‡
2 ” + 8 lauric acid	21/35‡
+ 8 myristic ”	10/20†
+ 8 palmitic ”	3/20
+ 8 stearic ”	7/19
+ 8 fatty acid like coconut oil*	13/14‡
+ 8 fatty acid like butter*	2/19
10% lauric acid	0/5
10 myristic ”	0/10
10 palmitic ”	0/7
10 stearic ”	0/9
10 fatty acid like coconut oil*	0/11

* Fatty acids like coconut oil were mixed to contain 64% lauric acid, 22% myristic acid, 12% palmitic acid, and 2% stearic acid. Fatty acids like butter were mixed to contain 2.5% lauric acid, 12.5% myristic acid, 37.5% palmitic acid, 12.5% stearic acid, and 35% oleic acid.

† $p < .01$ and

‡ $p < .001$ in comparison with corresponding results from 2 and 4% corn oil alone.

linoleic acid, but olive oil, which has about 75% oleic acid, fails to produce encephalomalacia, whereas lard, which contains about 40% oleic acid, balanced by an approximately equal amount of saturated fatty acids, does provoke cerebellar pathology. Similarly, ability of coconut oil to enhance appearance of encephalomalacia seems to be related to its high content of lauric and myristic acids, whereas butter, having mostly a mixture of oleic, palmitic, myristic, and stearic acids, has no such effect.

Summary. Corn oil or lard from which tocopherol had been removed, promoted chick encephalomalacia, whereas coconut oil, butter, linseed oil, and cod liver oil did not produce symptoms, and olive oil had a questionable effect. Dietary combinations of 2% corn oil with either 8% coconut oil, lauric acid, myristic acid, or a mixture of saturated fatty acids like coconut oil significantly increased the incidence of encephalomalacia over 2% corn oil alone, while 6% linseed oil, cod liver oil, or oleic acid inhibited the effect of 4% corn oil. Olive oil, butter, fatty acids like butter, palmitic acid, and stearic acid had no net effects upon the incidence of encephalomalacia induced by corn oil. The intake of linoleic acid appears to be a primary factor in the etiology of encephalomalacia, but some of the other fatty acids may secondarily increase or decrease this effect.

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Received August 12, 1959. P.S.E.B.M., 1959, v102.

A Method for Detection and Estimation of Penicillin in Poliomyelitis Vaccine.* (25255)

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During the manufacture of poliomyelitis vaccine, penicillin is introduced into the tissue culture medium at a concentration of 200 units/ml. Most of this penicillin is either removed or degraded during the processing of the vaccine. In recent medical literature there has been occasional reference to development of symptoms suggestive of penicillin sensitivity as a result of injecting poliomyelitis vaccine. However, data obtained during field trials in which this vaccine was tested, as well as experiences with subsequent mass immunizations, have shown that the incidence of sensitivity reactions is extremely low. Only 0.4% of subjects vaccinated in field trial studies, showed reactions to the vaccine. If only major reactions are tabulated, the incidence decreases to 0.004%(1). Although most of the penicillin introduced into poliomyelitis vaccine is removed or degraded to form microbiologically inactive products, it is of interest to determine residual pencillin in the finished vaccine. Microbiological assay indicated that residual penicillin would be present at low concentration. Because of this low concentration and because of presence in the vaccine of preservatives that interfere with microbiological assay, special precautions were necessary in developing a method that is free from interference and is capable of detecting 0.001 unit of penicillin or one part/billion in the finished vaccine. The method involves extraction of penicillin from the vaccine, followed by paper chromatographic analysis of the extract. Concentration of penicillin in the sample is calculated by comparing the bioautographic zones of inhibition produced by the sample with zones produced by penicillin standards.

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* Preliminary report presented at Fed. Am. Soc. for Exp. Biol., Atlantic City, Apr. 1959.

Methods. Known quantities of penicillin were chromatographed on dry Whatman #1 paper (19 cm × 46.5 cm) which had been buffered at pH 6.2 with 0.75 M phosphate buffer. During periods of high humidity, more consistency in zone sizes was obtained by applying penicillin standards in a 50% aqueous acetone solution. After evaporation of the solvent used in applying the penicillin sample, the sheet was developed for 16 hours by descending chromatography in a system consisting of diethyl ether saturated with water. At the end of development, the sheet was air dried and placed for 10 to 15 minutes on agar plate at pH 6.5 which had been seeded with *Bacillus subtilus,* ATCC 6633. After removal of the papergram, the plate was incubated 4 hours at 37°C and was left overnight at room temperature. Zones of inhibition due to penicillin, which had diffused from the papergram into the agar plate, were clearly visible and were photographed for a permanent record. Further details concerning the chromatographic and bioautographic procedure may be obtained from Bird and Pugh (2). To extract the penicillin, a sample of 100 ml of vaccine was adjusted to pH 2.2 and

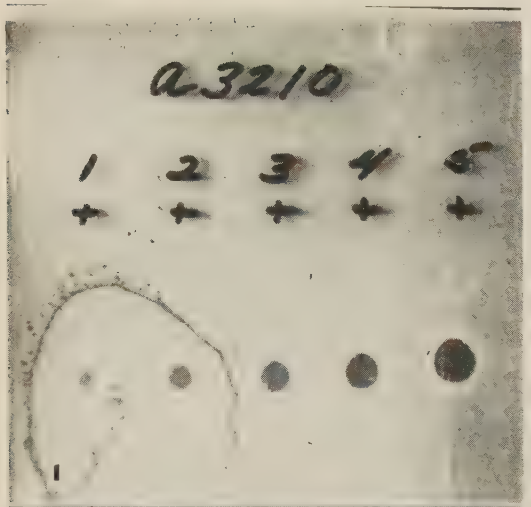

FIG. 1. Bioautograph of penicillin G. Lane 1, 0.0025 unit; Lane 2, 0.005 unit; Lane 3, 0.0075 unit; Lane 4, 0.01 unit; Lane 5, 0.02 unit. The plus signs indicate point of sample application.

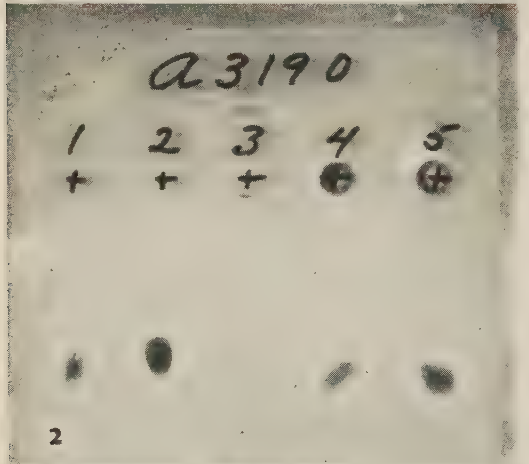

FIG. 2. Bioautograph of penicillin recovery from poliomyelitis vaccine. Lane 1, 0.005 unit penicillin G; Lane 2, 0.01 unit penicillin G; Lane 3, vaccine extract (100 μl) eq. to 5 ml vaccine; Lane 4, vaccine extract (100 μl) eq. to 5 ml vaccine to which 0.001 unit penicillin/ml had been added; Lane 5, vaccine extract (150 μl) eq. to 7.5 ml vaccine to which 0.001 unit penicillin/ml had been added.

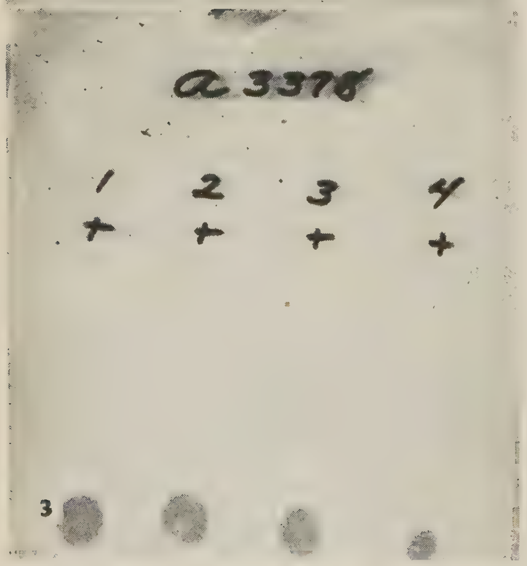

FIG. 3. Bioautograph of penicillin recovery from poliomyelitis vaccine. Lane 1, vaccine extract (10 μl) eq. to 0.5 ml vaccine to which 0.05 unit penicillin/ml had been added; Lane 2, 0.025 unit penicillin G; Lane 3, vaccine extract (5 μl) eq. to 0.25 ml vaccine to which 0.1 unit penicillin G/ml had been added; Lane 4, 0.0125 unit penicillin G.

was immediately extracted 3 times with 25 ml aliquots of cold anhydrous diethyl ether, (analytical grade). The ether extracts were withdrawn from the aqueous phase and were combined in presence of 1 ml of 0.1 M phosphate buffer at pH 7. Addition of the buffer at pH 7 insured stability of the penicillin in ether extracts. The procedure described may be carried out in a chillroom or in the laboratory at room temperature if the vaccine is kept cold. Ether-buffer mixture was concentrated under reduced pressure to the buffer phase. The concentrate of ether extractable material was adjusted to 2 ml by addition of acetone. Aliquots of this extract containing 0.03 unit or less of penicillin were chromatographed, following the procedure described for penicillin. The penicillin content of the vaccine was calculated by comparing zone sizes of the unknown samples with zone size of a known quantity of penicillin which had been chromatographed on the same sheet.

Results. A bioautograph which had been obtained from a chromatogram of penicillin is shown in Fig. 1. The antibiotic had been applied in the respective lanes at levels ranging from 0.0025 unit to 0.02 unit. Although a zone of inhibition is observable for 0.0025 unit of penicillin, this minute quantity is not routinely detectable. Consequently, 0.005 unit has been set as the minimum detectable level by the procedure described. If an amount of penicillin standard greater than 0.03 unit is chromatographed, it is difficult to use these zones for estimation of the antibiotic in an unknown sample. Zone sizes obtained at these higher concentrations of penicillin do not show the proportionate differences between concentrations that are observed at lower levels. The most accurate comparison of zone size between the standard and unknown sample is obtained in a range of 0.0075 unit to 0.03 unit of penicillin.

A bioautograph of a chromatogram on which a vaccine extract had been chromatographed is shown in Fig. 2. Since this vaccine contained less than 0.001 unit of penicillin/ml, this concentration of antibiotic was added to the vaccine and an extract prepared and chromatographed as described in the procedure. It is apparent from Fig. 2 that this concentration of penicillin is recoverable and detectable under conditions of this procedure.

It will be observed in Fig. 2 that zones of inhibition occur at the origin of each lane on which the vaccine extract was applied. Although the identity of these inhibitory materials has not been defined, this inhibition is probably due to the preservative, thimersol (Merthiolate), contained in the vaccine and perhaps to minute amounts of dihydrostreptomycin. The zones observed at the origin are not due to penicillin as evidenced by recovery data and also by the effect of penicillinase on the bioautograph. If penicillinase is added to the agar prior to pouring the plate for the bioautograph, the inhibitory material which has migrated from the origin is destroyed; however, the inhibitory material remaining at the origin is unaffected by the enzyme. In both Figs. 2 and 3, the migrating material extracted from the vaccine shows the same R_f value as the penicillin standards. Additional evidence for identifying the migrating material as penicillin is provided by the effect of penicillinase.

The results of another recovery study are shown in Fig. 3. Penicillin was added at a concentration of 0.05 unit and 0.1 unit/ml to 100 ml aliquots of vaccine. Aliquots of the respective vaccine extracts containing equivalent amounts of penicillin were chromatographed adjacent to 2 levels of penicillin. As seen in Fig. 3, excellent recovery of these levels of added penicillin was achived as indicated by similarity in zone sizes.

It has been possible to determine by our method that some lots of finished vaccine contain less than 0.001 unit of penicillin, while other lots contain a concentration of penicillin between 0.001 unit and 0.5 unit/ml.

Summary. A method is described for determining penicillin in poliomyelitis vaccine. This method consists of acidifying the vaccine, extracting the penicillin into diethyl ether, removal of ether by vacuum distillation and transfer of penicillin into a buffer. The penicillin is determined by standard paper chromatographic and bioautographic technics. This method is capable of detecting 0.001 unit of penicillin/ml finished vaccine.

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Received August 13, 1959. P.S.E.B.M., 1959, v102.

Some Pharmacological Observations on Tranylcypromine (SKF *trans*-385); A Potent Inhibitor of Monoamine Oxidase.* (25256)

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Zeller *et al.*(1) have demonstrated iproniazid (1-isonicotinyl-2-isopropylhydrazine) to be an inhibitor of monoamine oxidase. Shore and Brodie(2) and Brodie and Shore(3) have reported on the pyrexia, central nervous system (CNS) stimulation and sympathomimetic effects produced in rabbits pretreated with iproniazid followed by reserpine. These effects included increased motor activity and alertness, dilation of the pupil, increased heart rate and piloerection, and were attributed to the action of iproniazid as an *in vivo* inhibitor of monoamine oxidase. We have studied the effects produced by a new and potent *in vivo* inhibitor of monoamine oxidase, (tranylcypromine; 2-phenylcyclopropylamine hydrochloride); and compared its activity with both iproniazid phosphate and *d*-amphetamine sulfate.

Method. The test procedure employed was similar to that described by Brodie and Shore (3), and Horita(4). Several groups of rabbits were pretreated with a range of doses of either iproniazid (25 to 100 mg/kg), tranylcypromine (0.1 to 2 mg/kg), or *d*-amphetamine (1 to 5 mg/kg). Reserpine was administered at various intervals *thereafter* and rectal temperature recorded in an attempt to measure pyrexia at the time of *peak* drug action. The animals were also observed for signs of CNS stimulation. The potency of tranylcypromine relative to that of iproniazid was determined in 3 groups of 6 rabbits each. Groups of rabbits were treated with various doses of either compound. Reserpine was then administered at the time of peak drug action as previously determined. A regression line was obtained by plotting the per cent of rabbits exhibiting rectal temperatures of 42°C or higher against log dose. The dose which caused a rise of rectal temperature of 42°C or higher in 50% of rabbits (ED_{50}) and 95% fiducial limits were determined by the method of Litchfield and Wilcoxon(5). The durations of action of both tranylcypromine and iproniazid were determined by administering ED_{50}s of these compounds to groups of 6 rabbits. This was followed by administration of reserpine at various intervals thereafter. All compounds were investigated in rabbits by the intravenous route of administration. The dose of reserpine employed in all cases was 6 mg/kg. Rectal temperatures were determined with a thermistor thermometer. A converse series of experiments was also performed in which rabbits were first treated with reserpine and 2 hours later, when the animals were deeply sedated, either iproniazid, tranylcypromine or *d*-amphetamine was tested for its ability to arouse the rabbits from their reserpine stupor.

Results. Tranylcypromine produced maximum pyretogenic effects when it was administered 6 hours prior to reserpine. Time of peak effect for iproniazid was 20 hours.

High doses of *d*-amphetamine administered 1 to 6 hours prior to reserpine failed to produce the typical syndrome of symptoms consisting of pyrexia and central excitement. Tranylcypromine was found to be approximately 250 times more potent than iproniazid in this test procedure. The regression lines obtained by plotting the per cent of rabbits

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* A preliminary report was presented at Federation Meeting, Atlantic City, N. J., April, 1959.

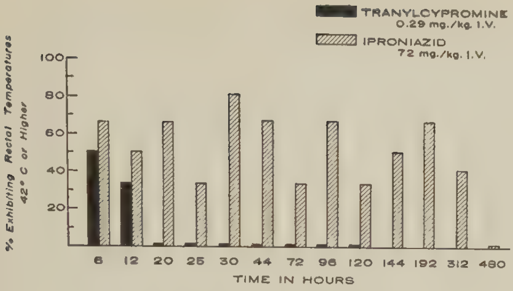

FIG. 1. Duration of action of both tranylcypromine and iproniazid in rabbits.

exhibiting rectal temperatures of 42°C or higher against log dose did not deviate from parallelism significantly. The duration of action of tranylcypromine and iproniazid is shown in Fig. 1. Observe that the duration of action of tranylcypromine is considerably shorter than that of iproniazid. The effects had subsided within 20 hours following its administration. In contrast, an equieffective dose of iproniazid was of extremely long duration lasting for as long as 13 days, although the effects were no longer apparent after 20 days. It should be emphasized that iproniazid *per se* at a dose of 100 mg/kg failed to produce any overt side effects, and tranylcypromine in doses as high as 5 mg/kg produced only moderate mydriasis in nonreserpinized rabbits. None of the compounds produced a significant rise in rectal temperature when given alone.

In the converse experiments in which either tranylcypromine, iproniazid, or *d*-amphetamine sulfate was studied for ability to arouse rabbits from their reserpine stupor, iproniazid was ineffective in doses up to 100 mg/kg(3). d-Amphetamine sulfate was effective as an analeptic against reserpine only in doses (2 mg/kg or higher) which produced central nervous system stimulation. Tranylcypromine effectively antagonized the reserpine-induced stupor at a dose of 5 mg/kg. Furthermore, this analeptic effect of tranylcypromine lasted for as long as 24 to 48 hours.

Discussion. Tranylcypromine appears to have a dual component of action. When administered to rabbits *before* reserpine it produced central excitation and pyrexia, and this effect is assumed to reflect the action of this compound as an inhibitor of monoamine oxidase. The results obtained in these experiments suggest that tranylcypromine is a relatively short-acting inhibitor of monoamine oxidase. Furthermore, our results support the findings of Hess and co-workers(6) who reported on the extremely long duration of action of iproniazid as an inhibitor of monoamine oxidase. It was of particular interest that these same workers suggested the possibility that iproniazid may act by destroying the enzyme monoamine oxidase. In this connection, it is tempting to speculate from our data that it takes at least 13 days for the resynthesis of monoamine oxidase to reach pre-iproniazid levels. Both tranylcypromine and d-amphetamine produced an analeptic effect in rabbits deeply sedated with reserpine. However, there were significant differences between these two compounds. The analeptic action of tranylcypromine was of much longer duration than that of *d*-amphetamine; in addition, tranylcypromine antagonized the reserpine-induced depression in doses which produced no overt signs of CNS stimulation.

Summary. Treatment of rabbits with tranylcypromine followed by the injection of reserpine produces central nervous system stimulation and a marked rise in rectal temperature. The effects produced by tranylcypromine are assumed to reflect the activity of this agent as an inhibitor of monoamine oxidase.

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Received August 14, 1959. P.S.E.B.M., 1959, v102.

Serologic Analysis of Ehrlich Tumor-Cell Induced Ascites Fluid.* (25257)

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It has been reported that viral antibodies can be found in ascites fluid induced in mice by Sarcoma 180(1) or Ehrlich tumor cells (2). Munoz(3) was able to induce large quantities of peritoneal fluid in mice by injection of Freund's adjuvant. The fluid was shown to contain antibodies against certain specific albumins used as immunizing agents; however, Munoz reported that only 50 per cent of the mice responded with an ascites fluid when injected with Freund's adjuvant. Recently Lieberman *et al.* reported(4) that intraperitoneal injection into mice of *S. aureus* mixed with Freund's adjuvant resulted in all of the mice producing an ascites fluid. Since large quantities of peritoneal fluid can be obtained regularly in mice injected intraperitoneally with Ehrlich tumor cells, it was of interest to serologically analyze the fluid to learn if the ascites could be employed as source for large quantities of various humoral components of mouse origin.

Materials and methods. In all experiments adult albino mice (*Mus musculus*) of mixed gender weighing 25-35 g each were employed. Mice were immunized by intraperitoneal injection of heat-killed *S. typhimurium*. The injection schedule was 0.1 ml of vaccine administered at intervals of one week until 3 injections were completed. Immediately following last injection the mice were intraperitoneally injected with 0.25 ml of a heavy suspension of ascites cells taken from peritoneal cavities of infected mice. Another group of mice were injected only with the tumor cell suspension. The Ehrlich tumor cell challenged mice were sacrificed after 10 days at which time all animals used were markedly distended with ascites. The blood as well as the ascites fluid was collected and pooled. The average yield of fluid/mouse at this time was 20-30 ml. The average yield of blood following severance of neck vessels was 2-3 ml/mouse. The blood was allowed to clot 2 hours at room temperature; the clot was rimmed and removed by centrifugation at high speed in a refrigerated centrifuge. Cells in the ascites were removed by mild centrifugation in the cold. Aliquots of both serum and cell-free ascites fluid were then recentrifuged at 25,000 g for 15 minutes. The serum and ascites fluid were assayed for whole complement (C′) activity employing the micro-Kolmer method(5). Properdin assays were performed using the zymosan technic of Pillemer *et al.*(6). Other serologic tests were performed using standard technics(7). Electrophoretic patterns were obtained by paper strip technic employing a Spinco Model R apparatus. Total protein concentrations were determined by micro-Kjeldahl procedures.

Results. The data in Table I indicate that neither mouse ascites nor serum contain normal agglutinins for human or sheep red blood cells(8). The ascites from mice immunized against *S. typhimurium* contained agglutinating antibodies to the immunizing agent although the titer was lower than the serum titer. There was no detectable properdin in the ascites from immunized or nonimmunized mice, but serum properdin levels were within limits reported by others(9). The serum and ascites were tested for whole C′ titer and contained less than 3 units/ml; both fluids had similar anticomplementary properties which could be removed by 1:4 dilution in buffer of serum or ascites fluid.

The data in Table II show total protein concentrations of serum and ascites. In addition the concentration of the various protein components of the 2 fluids is shown. Gamma component of globulin in the ascites from immunized mice constitutes 37% of total protein while the same component in the ascites from the nonimmunized mice comprises 29%. The major difference between the ascites from immunized and nonimmunized mice appears

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* Aided by grant from Amer. Cancer Soc.

[†] P.H.S. Research Fellow of Nat. Inst. of Allergy and Infectious Diseases.

TABLE I. Serological Analysis of Mouse Serum and Ascites Fluid from Ehrlich Tumors.*

	Mouse serum		Mice with Ehrlich ascites tumor			
			Nonimmunized mice		Immunized mice	
	Normal	Immunized	Serum	Ascites	Serum	Ascites
Human RBC agglutinin titer	neg.		neg.	neg.		
Sheep " " "	"		"	"		
Properdin titer (units/ml)	10	10	10–20	<4	10–20	<4
Whole C′ titer (units/ml)	<3	<3	<3	<3	<3	<3
Anti C′ property removed at	1:4	1:4	1:4	1:4	1:4	1:4
S. typhimurium agglutinin titer	neg.	1:8192	neg.	neg.	1:8192	1:2048

* All results similar with specimens centrifuged at 25,000 g or low speed.

to be in the beta globulin component. It is apparent from the results that the protein synthesizing capacity is not impaired as a result of tumor cell growth. To the contrary, it appears that compensatory synthesis occurred to make up the protein content of the ascites.

Discussion. Intraperitoneal injection of Ehrlich tumor cells into mice regularly induced an ascites which amounted to 20-30 ml/mouse when the fluid was removed from animals 10 days post-challenge. The ascites and serum did not contain any normally occurring agglutinins for human or sheep erythrocytes or for *S. typhimurium*. The ascites contained specific agglutinating antibody to *S. typhimurium* when the mice were immunized against this agent. The agglutinin titer of the ascites was lower than that present in serum of immunized mice but of sufficiently high titer to be employed for study of antibody reactions.

The ascites did not contain any detectable properdin activity at the dilution of fluid (1:4) employed in the assay. The whole C′ titers of serum and ascites were of the same magnitude, that is, less than 3 units/ml in each case. Both fluids were anticomplementary, however this effect could be overcome by 1:4 dilution in both cases.

Values for concentration of serum and ascites protein constituents were obtained from pooled fluid samples. This design was adopted to decrease the error involved in an experiment of this type.

Concentrations of protein components in serum from normal or specifically immunized mice are very similar to those reported by Clausen *et al.*(10). Serum obtained from mice free of the neoplasm exhibited a high concentration of beta globulin as compared to alpha and gamma fractions. Clausen *et al.* (10) reported that serum from (CBA × DBA/2) F_1 hybrid mice, infected with plasma cell leukemia, showed increased amounts of beta globulin components and decreased amounts of gamma components while no significant change occurred in the alpha components. In the present investigation a loss of beta globulin from the serum appeared to occur as a result of Ehrlich tumor cell infection.

TABLE II. Paper Strip Electrophoretic Analysis of Mouse Serum and Ascites from Ehrlich Tumors.

Material	No. of specimens pooled	Total protein	Albumin	Globulin		
				α	β	γ
Normal mice serum‡	12	6.78*	2.95	1.22	1.81	.8
Immunized mice serum†	12	5.7	2.6	1.0	1.4	.7
Mice with Ehrlich ascites tumor						
Nonimmunized mice serum	15	4.9	2.11	.7	.6	1.5
Nonimmunized mice ascites§		3.1	.9	.2	1.1	.9
Immunized mice serum	14	6.45	3.5	.7	1.5	.75
Immunized mice ascites		3.5	1.36	.39	.44	1.31

* g %.
† Immunized by 3 intraper. injections of heat-killed *S. typhimurium* given one week apart.
‡ Avg yield of serum/mouse .5-1.5 ml.
§ " " " ascites/mouse 20-30 ml.

It is of interest to note that serum of non-immunized mice infected with tumor cells showed less beta and more gamma globulin protein than the ascites fluid obtained from the same group of mice. In contrast, the serum of immunized mice infected with tumor cells showed more beta and less gamma globulin protein than the ascites fluid obtained from the same pool of mice.

It is apparent that by suitable fractionation of ascites fluid large amounts of purified antibody, if not properdin, can be obtained for study of mouse serologic reactions.

Summary. Ascites fluid induced in mice by intraperitoneal injection of Ehrlich tumor cells contained specific antibody to *S. typhimurium* when mice were immunized against this organism. The serum and ascites were identical in their anticomplementary properties and neither contained detectable complement. The serum had normal properdin levels, but the ascites did not contain detectable properdin under the assay conditions employed.

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Received August 25, 1959. P.S.E.B.M., 1959, v102.

Effects of Nitrogen Mustard N-Oxide, X Rays, and Cortisone on Yoshida Sarcoma Metastasis Development. (25258)

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At present, the most effective treatment for cancer is complete removal of malignant tissue by means of surgery. Despite the great value of this method, however, its usefulness is unfortunately limited by the difficulties involved in locating metastases. This particular limitation does not apply to carcinostatic agents, since their effects are systemic. Nevertheless, the mode of action of such therapeutic agents is but poorly understood, and many problems remain to be solved. For example, we have observed that the use of carcinostatic agents has sometimes been followed by an increase in tumor size or by the production of metastases, thus multiplying the problems connected with surgery.

Materials. An outbred strain of Gifu male rats weighing about 100 g was used in this investigation. Eight million cells of a 7-day-old Yoshida ascites tumor were transplanted subcutaneously into the right axillary area. Three weeks after tumor implantation, the animals were autopsied and examined for metastases. Certain batches of injected cells were taken from a tumor which had been made resistant to the carcinostatic effects of nitrogen mustard N-oxide ("NMO").† This tumor, which was kindly supplied by Dr. I. Hirono(1), had been made resistant to NMO

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† The NMO used was supplied under trade-name NITROMIN by Yoshitomi Pharmaceutical Industries, Osaka, Japan. The chemical name for this brand of NMO is methylbis(2-chlorethyl)amine N-oxide hydrochloride.

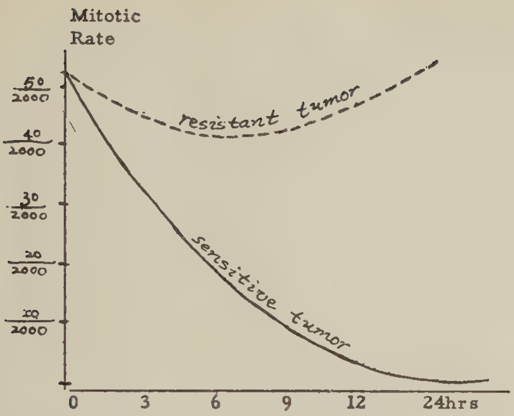

FIG. 1. Effect of NMO on the ascites tumor cell.

as follows: After tumor transplantation, subcutaneous doses of 50γ of NMO were given daily. The effects of this dosage on survival time, cell count, and mitotic rate gradually diminished to nil. After each cessation of effect, the dosage was increased, first to 100γ, then to 250γ, and finally to 500γ. About 70 to 100 days were required in order to develop a tumor resistant to a daily dosage of 500γ of NMO. Resistance of a tumor to NMO was tested by injecting a dose of 5 mg/kg body weight. The effect of this dose on mitotic rate is shown in Fig. 1. When the tumor is sensitive, the mitotic rate falls consistently, and the tumor does not grow. When the tumor is resistant, however, the mitotic rate temporarily undergoes a slight change, cell division being abnormal 3 and 6 hours after NMO injection, but recovering after 9 hours. In the absence of NMO, the results of transplanting NMO-sensitive and NMO-resistant tumors are essentially identical. When 5 mg/kg/day of NMO is administered for 21 days, however, a subcutaneously implanted resistant tumor containing 8 x 10^6 cells enlarges to as much as 1.5 x 1.2 cm.

Results. Four general classes of treatment were studied: A. NMO for NMO-sensitive tumors; B. NMO for NMO-resistant tumors; C. X rays; D. cortisone. Each general class was divided into 4 specific types of treatment: 1. implantation of tumor cells, without treatment (controls); 2. treatment begun one week *after* implantation of tumor cells, and continued for one week; 3. treatment begun one week *before* implantation of tumor cells, and continued only for that week; 4. treatment begun one week *after* implantation of tumor cells, and continued for two weeks, till autopsy. The effects of the 16 individual types of treatment are summarized in Table I.

A. NMO for NMO-sensitive tumors. NMO was given in a dosage of 5 mg/kg/day. Types 1 and 4 resulted in only slight metastases, but types 2 and 3 produced remarkable ones. The results of type 2 suggest that the development of metastases is promoted by amounts of NMO insufficient to be carcinostatic. The results of type 3 demonstrate that the effect of NMO on the host is an important factor in the production of metastases. The results of type 4 indicate that the development of metastases can be inhibited by sufficiently large amounts of NMO.

B. NMO for NMO-resistant tumors. The dosage of NMO was the same as for NMO-sensitive tumors. All 4 types gave results similar to those produced by type A1. Such results indicate that NMO-resistant tumors have a limited capacity for forming metastases, regardless of the specific type of treatment within this general class. Since the effect of NMO on the host is presumably the

TABLE I. Effects of NMO, X rays, and Cortisone on Metastasis Development.

	Nitrogen mustard N-oxide						X rays			Cortisone		
	Sensitive tumor			Resistant tumor								
Type	No. of rats	Tumor	Metastases	No. of rats	Tumor	Metastases	No. of rats	Tumor	Metastases	No. of rats	Tumor	Metastases
1	11	2+	2+	11	2+	2+	11	2+	2+	11	2+	2+
2	12	3+	3+	8	2+	2+	8	2+	3+	12	2+	2+
3	12	3+	3+	12	2+	2+	8	2+	3+	18	2+	3+
4	12	—	1+	7	2+	2+	16	—	1+	10	2+	2+

Metastases: 1+, metastases to regional lymph glands and mediastinal lymph glands; 2+, metastases to abdominal lymph glands; 3+, metastases to various organs.

Tumor: —, none; 1+, less than 1 cm diameter; 2+, 1 to 2 cm diameter; 3+, more than 2 cm diameter.

same whether the tumor is sensitive or resistant, the phenomena produced by this class of treatment cannot be ascribed to host resistance. Evidently NMO-resistant tumors have little tendency to liberate cells into the blood stream.

C. X Rays. A dose of 100 r was given to the whole body every other day. Each type gave results essentially the same as those produced by the corresponding type in class A. These results indicate that the effects of X rays are closely parallel to those of NMO on an NMO-sensitive tumor. Whereas the NMO-sensitive tumors treated with NMO merely regressed, however, the tumors treated with X rays became necrotic.

D. Cortisone. The dosage was 50 mg/kg/day. Types 1, 2, and 4 gave results essentially the same as those produced by type A1, but type 3 produced remarkable metastases. These results suggest that cortisone does not promote the release of tumor cells into the blood stream, but does have a strong effect on the host.

Discussion. Metastasis production by means of cortisone was first reported by Agosin and his associates(2), and later confirmed by other investigators(3,4). Agosin's group concluded that this type of metastasis production is associated with inhibition of host resistance. Further consideration of this phenomenon has led to the development of "soil hypotheses" regarding susceptibility of the "soil." Metastasis production by means of local treatment with X rays has been explained as a transient local disturbance of the host-tumor relationship, probably facilitating entry of tumor cells into the blood stream(5-8). These reports suggest that chemical or physical carcinostatic agents have two types of effects on metastasis production: first, effects on the tumor itself, releasing tumor cells into the blood stream; second, effects on the host or on the organs invaded by the tumor.

The results of the present investigation support these conclusions. Evidently NMO has both types of effects on metastasis production, and so also do X rays. Cortisone, however, presumably has an effect only on the host. Considering the ability of carcinostatic agents to produce metastases when treatment is not continued long enough, it is apparent that clinical use of carcinostatic agents cannot be expected to provide satisfactory results if the doses employed are insufficient.

Summary. 1. If a subcutaneously implanted Yoshida sarcoma is sensitive to nitrogen mustard N-oxide ("NMO"), pre-treatment or short-time post-treatment with NMO produces remarkable metastases. The severity of these metastases following pre-treatment with NMO suggests a strong effect on the host. 2. Treatment of NMO-resistant tumors with NMO does not promote the development of metastases, even with pre-treatment or short-time post-treatment. This indicates that NMO-resistant tumors do not liberate cells to form metastases even when the host is under the influence of NMO. 3. Treatment of tumors with X rays produces essentially the same results as treatment of NMO-sensitive tumors with NMO does. In both instances, diminution of host resistance appears to be an important factor in promoting the development of metastases. 4. Pre-treatment of tumors with cortisone promotes the development of metastases; post-treatment, whether short-time or extended, has no such effect. Evidently the chief action of cortisone is on the host, increasing the ease with which metastases are formed. 5. Post-treatment with either NMO or X rays for an extended period inhibits the formation of metastases, and causes the tumor itself to regress. This observation suggests that carcinostatic agents should be used clinically only in large enough doses.

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Received August 28, 1959. P.S.E.B.M., 1959, v102.

Erythropoietic Activity in Plasma and Urine of Dogs After Bleeding.*
(25259)

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Since Reissmann's experiments(1) on parabiotic rats established humoral regulation of erythropoiesis, numerous workers(2-5) demonstrated erythropoietic activity in plasma and urine of several mammalian species made hypoxic or anemic. The only material available for chemical characterization of erythropoietic factors is plasma of rabbits treated with phenylhydrazine or active urine of some anemic patients(6-10). More recently, Hodgson *et al.*(11) proposed that urine of anemic rabbits may serve as a source of erythropoietin. No demonstration has been made of an erythropoietic factor in plasma of dogs after bleeding. The present study demonstrates presence of erythropoietin in plasma and urine of bled dogs.

Material and methods. Six dogs weighing between 12 and 26 kg were bled once or twice daily for 5 to 6 days to hematocrit values from 5 to 11%. Blood volumes were maintained by replacing amount of blood drawn with equal volume of 6% dextran in saline.‡ Plasma samples were frozen and stored until used. Boiled filtrates of normal and anemic plasma were prepared by Borsook's method (12). The material was adjusted to pH 5.5 with 1N HCl, boiled 15 minutes, and filtered. The precipitate was washed with equal volume of distilled water, the wash added to filtrate and samples adjusted to initial volume of plasma. Urine was frozen immediately after collection and assayed without further treatment. Erythropoietic activity of plasma and urine was measured by Fe^{59} red cell incorporation assay(13), using starved female rats of Long-Evans strain weighing 150 to 200 g. After 24 hours starvation each rat received 2 cc plasma or urine subcutaneously daily for 2 successive days. Twenty-four hours after second injection, Fe^{59} (30,000 to 100,000 c/min.; specific activity 2 $\mu c/\gamma$) combined with rat plasma was injected intraperitoneally. Eighteen to 20 hours later, a sample of blood was obtained from aorta and counted. Five to 10 rats were used for each determination. Controls consisted of both uninjected rats and groups receiving normal plasma. Erythropoietic activity of plasma samples from the same dog obtained at different states of anemia was compared to normal plasma obtained by first venesection. Hematocrits were measured in Wintrobe tubes by centrifugation at 3,000 rpm for 30 minutes. Platelet determinations were made by direct technique using Rees-Ecker solution as diluent and leukocytes with 2% acetic acid.

Results. Plasma of bled dogs contains a factor capable of stimulating Fe^{59} red cell uptake in starved rats (Fig. 1). When hematocrit was above 16%, no activity was demonstrated in plasma. Although activity was present in plasma from dogs bled to hematocrit of 12% (dog 4: 11.4% uptake and dog 6: 15.4%), a more striking response was obtained when the hematocrit was 9% or less (dog 2: 30% uptake, dog 4: 30.9%).

Urine samples from 3 dogs were assayed (Fig. 2). Activity was obtained from urine of 2 dogs whose hematocrits were 8% and 9%: 26.9% Fe^{59} uptake after injection of urine from dog 5 and 17.8% after urine injection from dog 6. Urine from a third dog whose hematocrit was 11% was inactive: 4.2% uptake. This urine was also inactive after dialysis for 24 hours at 4°C (4.8% uptake).

The plasma retained activity after remaining at room temperature for 24 hours but plasma and urine activity often decreased after weeks of storage in frozen state (Table

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* This study is based on work performed under contracts with U. S. Atomic Energy Comm.

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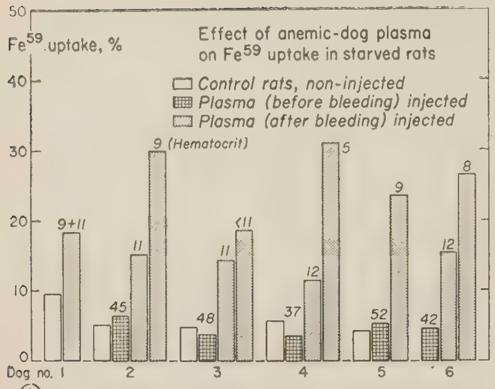

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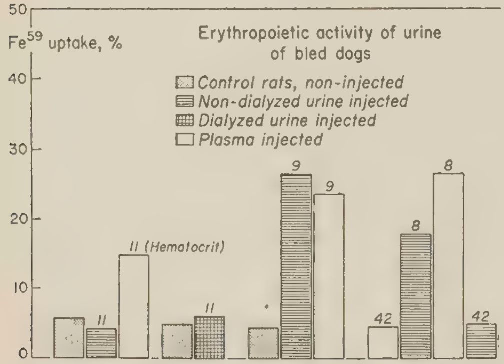

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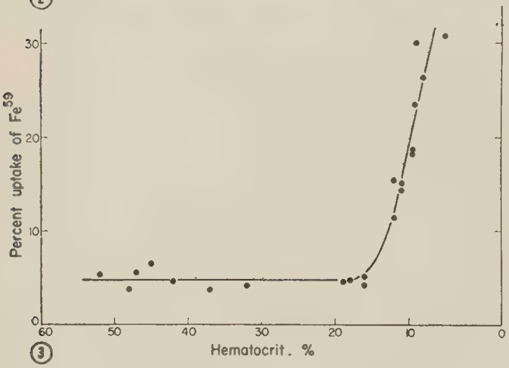

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FIG. 1. Hematocrit value at time of withdrawal of plasma is given at top of each column.

FIG. 2. Erythropoietic activity of urine sample compared with correspondent plasma. Figure at top of each column indicates hematocrit value at time when material was collected.

FIG. 3. Values of erythropoietic activity of plasma in regard to hematocrit measurements.

I). Plasma activity was partially destroyed by boiling 15 minutes (Table II).

Injection of active erythropoietic plasma for 2 consecutive days did not alter number of white cells or platelets of recipient rat.

Discussion. When sufficient anemia is produced, erythropoietic activity appears in dog plasma, but the dog is not as responsive to bleeding as rabbit, rat, or monkey. In these species, others(2-4,14) have demonstrated erythropoietic activity when the hematocrit is 20% and hemoglobin is 6 g%. However, assay methods were different from that used in our investigation. It is difficult to demonstrate activity in plasma of dogs because it is necessary to produce severe anemia, which requires frequent venesections and careful maintenance of blood volume with plasma substitute (Dextran) to avoid fatal shock.

TABLE I. Effect of Storage in Freezer (−18°C) on Erythropoietic Activity of Plasma and Urine.

Sample No.	Date of assay	% Fe^{59} uptake	No. recipient rats
Plasma 6.19	12/25/58	26.4 ± 5.2*	9
Controls†	"	4.6 ± 2.4	9
Plasma 6.19	1/ 9/59	21.9 ± 4.6	6
Controls	"	5.6 ± 1.4	6
Plasma 6.23	"	14.8 ± 4.3	6
Controls	"	5.6 ± 1.4	6
Plasma 6.23	1/23/59	8.2 ± 1.5	6
Controls	"	3.1 ± .6	6
Plasma 34.8	12/12/58	30.9 ± 4.9	10
Controls	"	5.7 ± .89	10
Plasma 34.8	2/20/59	15.5 ± 5.6	6
Controls	"	3.3 ± 1	8
Urine 36.19	12/25/58	17.8 ± 4.8	10
Controls†	"	4.6 ± 2.4	9
Urine 36.19	1/30/59	19.7 ± 9.4	7
Controls	"	4.6 ± .79	6
Urine 35.14	12/20/58	26.4 ± 8.2	10
Controls	"	4.3 ± .70	10
Urine 35.14	2/20/59	10.7 ± 4.3	6
Controls	"	3.3 ± 1	8

* Stand. dev.

† In these groups, rats inj. with normal plasma are used as controls; other control groups are uninj. rats.

TABLE II. Effect of 15 Minutes Boiling on Erythropoietic Activity of the Plasma.

Material inj.	Quantity inj. daily, cc	% Fe^{59} uptake	No. of recipients
Normal plasma	2	6.5 ± 3.6*	10
—after boiling	2	6.3 ± 6.2	"
''Anemic'' plasma	2	15.1 ± 3	"
—after boiling	2	10.6 ± 4.9	9
''Anemic'' plasma	2	30 ± 4.7	"
—after boiling	2	18.6 ± 6.8	10

* Stand. dev.

Fig. 3 shows that no activity was measured in plasma when hematocrit was 16% or more, some activity appeared when hematocrit reached 12%, and a sharp increase in erythropoietic stimulating factor occurred with lower hematocrits. The dose-response curve published by Garcia and Van Dyke(15) indicated that uptake of 30% required 5 times as much erythropoietin as uptake of 15%. Thus the erythropoietic factor response in the dog after bleeding is more striking than appears at first sight.

Four of the 6 dogs died from anemia; it is interesting that the most active plasma obtained was the last sample taken before death. When dogs were made severely anemic in 6 hours by replacing blood with dextran several times, no erythropoietic activity could be observed in plasma at the 6th hour.§ Possibly anemia of some duration is necessary to obtain demonstrable erythropoietin titer.

Like rabbits(11) and human beings(7,8), the dog secretes erythropoietin in urine when severe anemia exists. A very low hematocrit (9% or less) must be reached before activity is found in unconcentrated urine (Fig. 2). Since anemia of this degree is almost incompatible with life, this may explain why erythropoietic factor is seldom found in urine of anemic patients.

Erythropoietic factor may appear in urine as a result of glomerular filtration or tubular secretion. The latter mechanism is not unlikely since the role of dog kidney in erythropoietin production has received experimental support(16,17).

Boiling for 15 minutes reduces erythropoietic stimulating activity of plasma; Fe^{59} incorporation was reduced from 30% to 15% which is an 80% loss of activity according to dose-response curve. Stohlman(18) demonstrated a similar loss of activity when erythropoietically active rabbit plasma was boiled.

Active plasma and urine are obtainable from bled dogs and provide a source of erythropoietically active material for both biological and chemical investigation. It must be emphasized that this material is unstable even in frozen state. Lyophilizing it may prevent loss of activity.

Summary. Active erythropoietic factor is demonstrated in plasma and urine of bled dogs. A very striking erythropoietic factor response is obtained when severe anemia is produced. Activity is partially destroyed by boiling and by prolonged storage in freezer.

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Received March 19, 1959. P.S.E.B.M., 1959, v102.

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Effect of Phenethylbiguanide on Serum Inorganic Phosphate.* (25260)

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Following the preparation of an active insulin extract by Banting and Best(1), a rapid fall in inorganic phosphorus of blood was demonstrated in normal animals by Wigglesworth *et al.*(2), in rabbits after insulin convulsions by Winter and Smith(3), in human diabetics by Perlzweig and co-workers(4), and in rabbits, dogs, normal humans, and patients in diabetic coma by Harrop and Benedict(5). Their data offered further support to the prevailing theory of early twentieth century that there was a close participation of phosphate ion in metabolism of carbohydrate(6). Harrop and Benedict(7) likewise observed a drop in concentration of serum inorganic phosphate during active metabolism of ingested carbohydrate, and De Venanzi(8) later found that the effect of glucose does not take place in pancreatectomized animals but seems due to an insulin discharge determined by pancreatic stimulation. There are conflicting reports of the effect of sulfonylurea oral hypoglycemic agents on serum inorganic phosphorus. Renold *et al.*(9) report that there is no change in serum inorganic phosphate in human subjects after administration of carbutamide or tolbutamide, and De Venanzi(8) made the same observation in dogs treated with carbutamide. On the other hand, Goetz and associates(10) found that tolbutamide decreases the serum concentration of this ion. Similarly, in a study of the effect of chlorpropamide on serum inorganic phosphorus levels, no evidence of insulin-like action was reported by Stowers *et al.*(11) and by Izzo(12), while Dobson *et al.*(13) indicate that the effect of chlorpropamide is the same as that following administration of insulin. In marked contrast, Synthalin A or decamethylenediguanidine, the oral hypoglycemic compound synthesized by Frank and co-workers(14) was found to produce an increase in inorganic phosphorus of blood by Florence *et al.*(15). In our investigation, the action of phenethylbiguanide on this blood constituent in normal rabbits is reported. This biguanide compound, reported to possess marked oral hypoglycemic properties by Ungar and associates(16), was suspected of increasing serum inorganic phosphorus since it has been found to imitate the action of decamethylenediguanidine in many of its physiological effects.

Materials and methods. New Zealand white male rabbits weighing between 2 and 4 kg were fed Purina Rabbit Chow *ad lib.* except for the fasting period prior to experiments. Blood samples for glucose and phosphate analyses were obtained by heart puncture on unanesthetized animals and all injections were administered subcutaneously in the abdominal region, since response to oral hypoglycemic agent has been reported more consistent by this mode of administration(16). Quantitative estimation of serum glucose was made by the method of Folin and Wu(17), while phosphate was determined by the method of Fiske and Subbarow(18). A 0.9% saline solution was used for injection into control animals and was also used as the medium in which phenethylbiguanide solution was prepared. Both experimental and control animals represented in Table I were fasted 15 hours prior to withdrawal of first blood sample. Phenethylbiguanide (PEBG) was then administered at dosage level of 30 mg/kg of body weight to 6 experimental animals, while 6 control rabbits were given saline solution. Three hours later the second blood sample was obtained. In experiments in Table II, phenethylbiguanide induced convulsions were produced in rabbits. In these experiments, the same rabbits served as both control and ex-

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* Authors wish to thank Dr. Harvey S. Sadow, U. S. Vit. Corp., N. Y., for supplying phenethylbiguanide (DBI) and Eli Lilly Co., Indianapolis, Ind. for furnishing glucagon-free insulin used. Project supported in part by a grant-in-aid from Chicago Heart Assn.

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TABLE I. Response of Rabbits to Changes in Serum Inorganic Phosphorus and Glucose Levels.

Phenethylbiguanide (PEBG) treated animals

Mean mg % serum glucose				Mean mg % serum inorganic phosphorus			
Before PEBG	Stand. dev.	After PEBG	Stand. dev.	Before PEBG	Stand. dev.	After PEBG	Stand. dev.
144	33.7	86	16.6	6.96	1.06	8.31	1.05

Saline treated control animals

Before saline	Stand. dev.	After saline	Stand. dev.	Before saline	Stand. dev.	After saline	Stand. dev.
113	11.8	114	9.8	6.86	.96	6.79	.92

TABLE II. Effect of Phenethylbiguanide (PEBG) Induced Convulsions on Serum Inorganic Phosphorus and Glucose Levels of Rabbits.

Mean mg % serum glucose						Mean mg % serum inorganic phosphorus					
Before saline	S.D.	After saline, before PEBG	S.D.	After PEBG	S.D.	Before saline	S.D.	After saline, before PEBG	S.D.	After PEBG	S.D.
85	13.0	80	11.8	17	17.7	6.57	.72	6.60	.85	8.75	1.17

perimental animals. Four animals were fasted 48 hours, then a blood sample was taken and an injection of saline solution administered for control purposes. Three hours later a second blood sample was taken and an injection of phenethylbiguanide was administered at dosage level of 50 mg/kg of body weight. After another 3 hours fasting, a third blood sample was withdrawn and analyses were made on the 3 serum samples for glucose and inorganic phosphate. For comparative purposes, the 5 rabbits (Table III) were treated with glucagon-free insulin (U-40 Iletin) at a dose of approximately 15 U/kg of body weight, to produce insulin convulsions. Three hours elapsed between administration of insulin and withdrawal of second blood sample.

Results. In normal rabbits, phenethylbiguanide injected subcutaneously induces a decrease in blood sugar levels. This change in blood sugar is accompanied by significant increase in serum inorganic phosphorus. Insulin, on the other hand, produces a marked decrease in serum inorganic phosphate along with a decrease in serum glucose. This would suggest that the action of phenethylbiguanide is not mediated through extra insulin secretion. In its effect on serum levels of this ion, phenethylbiguanide then resembles the action of diguanidine, Synthalin A, rather than sulfonylurea hypoglycemic agents investigated. This observation was expected in that the biguanide resembles the diguanidine in many of its physiological actions.

Increase in serum inorganic phosphorus levels following treatment of animals with phenethylbiguanide is also consistent with inhibition of oxidative enzyme systems in the citric acid cycle by this compound observed by Steiner and Williams(19) and by Wick *et al.*(20). This inhibition suggests a decreased binding of high energy phosphorus, thus allowing inorganic phosphorus to escape into the blood stream. The divergence in action of insulin and phenethylbiguanide on serum inorganic

TABLE III. Response of Rabbits to Changes in Serum Inorganic Phosphorus and Glucose Levels following Administration of Insulin.

Mean mg % serum glucose				Mean mg % serum inorganic phosphorus			
Before insulin	Stand. dev.	After insulin	Stand. dev.	Before insulin	Stand. dev.	After insulin	Stand. dev.
84	18.5	10	9.2	6.14	.70	5.15	.68

phosphorus seems to invalidate use of the fall in serum concentration of this ion as an index of peripheral utilization of carbohydrate.

Summary. The normal rabbit, treated with hypoglycemic doses of phenethylbiguanide, shows a significant increase in serum inorganic phosphate levels. In this respect the action of phenethylbiguanide differs from that of insulin and sulfonylurea oral hypoglycemic agents, and closely resembles that of structurally related diguanidine, Synthalin A. Increase in serum inorganic phosphorus levels is consistent with inhibition of oxidative enzyme systems of the citric acid cycle in presence of phenethylbiguanide, which suggests that inorganic phosphorus might escape into the blood stream due to decreased binding of high energy phosphorus.

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Received June 1, 1959. P.S.E.B.M., 1959, v102.

Further Studies on 2060 Virus.* (25261)

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Previous reports have described isolation of an apparently new viral agent, designated 2060, and have presented the case for etiological relationship to mild, afebrile, coryzal illnesses(1-4). Antigenic relationship, but not identity to the JH virus was also noted. The latter agent was isolated independently by Price and was also thought to be the cause of similar illnesses in man(5-7). Both of these viruses appear to have characteristics in common, including rate of growth and metabolic requirements, stability, cytopathogenic effects and infectivity for the same hosts. Certain of these features of 2060 are described herein.

Methods. Cultures (TC) were prepared with 0.25% trypsin (Difco, 1-250) dispersed kidney cells from young adult male Rhesus monkeys, planted at a 1-400 dilution of cells packed at 800 rpm for 10 minutes and grown at 35°C in stationary tubes and prescription bottles with 0.5% lactalbumin hydrolysate (Nutritional Biochemicals) and 2% calf serum (Microbiological Associates) in Earle's solution. After 4 to 7 days they were washed twice and maintained with Mixture 199 or Eagle's medium (Microbiological Associates), or the 2 combined in equal parts, with care to

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* Supported by USPHS grant and Abbott Labs., North Chicago, Ill.

† Present address: Children's Hospital Research Fn., Cincinnati, O.

lower pH with CO_2 sufficiently so that it would remain within 7.0-7.2 at 35°C. Cell lines derived from human amnion and lung and kitten lung (obtained from Dr. C. Pomerat) and human kidney (Chang) were propagated in 199 or Eagle's with 10% horse serum and maintained for experiments as above. Stock virus, 2060 TC_{5-7}, was prepared in tube and bottle cultures inoculated with 0.1 ml/ml of TC fluid containing 10^{3-4} $TCID_{50}/0.1$ ml, harvested after 5 to 7 days growth at 35°C, clarified by centrifugation at 1500 rpm for 10 minutes, quick frozen and stored at –65° C. Infectivity ($TCID_{50}$) was determined by inoculating 0.1 ml of 10-fold dilutions of TC fluids into groups of 4 monkey kidney cultures. Readings were made at intervals up to 7 days and a culture was considered infected by appearance of characteristic cytopathology. Neutralization titers were determined by adding 10^3TCID_{50} of virus to equal quantities of 2 fold dilutions of inactivated sera (56°C, 30 minutes), incubating at 4°C for 30 minutes, and inoculating groups of 3-4 cultures with 0.1 ml of serum-virus admixture. Titers were calculated on the basis of initial dilution of serum inhibiting the development of cytopathogenic effects at 7 days.

Results. Growth in stationary cultures. To correlate yield of infectious virus with development of cytopathogenic changes in stationary cultures, a small infecting dose was used as shown in Table I. Increments in infectious virus and proportion of involved cells reached a plateau despite the presence of many unaffected cells. By replacement of maintenance medium virus titers could be sustained for several additional days until a majority of the cells had undergone complete disruption. In other experiments established cell lines, including human kidney, human amnion and lung and kitten lung, demonstrated no cytopathogenic effects and produced no infectious virus after inoculation of 2060.

TABLE I. Growth of 2060 TC_6 in Stationary MK Cultures.*

Days after infection	Degree of CP effect	Virus yield, $ID_{50}/0.1$ ml (log)
1	0	1.5
2	+	2.0
3	++	3.0
5	++ ++	3.5
7	++ ++	3.7

* Inoculum was 10^2TCID_{50}. Groups of 4 cultures were harvested at intervals shown.

TABLE II. Effect of Various Constituents of Eagle's Medium on 2060 Virus Yield in Monkey Kidney Cultures.

Maintenance media*	Virus yield, $ID_{50}/0.1$ ml (log) A	B
V + G + AA	4.0	3.0
V + G	3.8	
V + AA	3.2	2.0
G + AA	3.0	

* Cultures maintained 3 days in modified media prior to inoculation and same medium replaced at time of infection.

V, vitamin mixture, 1%; AA, amino acid mixture, 1%; G, glutamine, 200 mM, 1%.

Nutritional factors. Since virus yield and cytopathogenic effects were greater in monkey kidney cultures maintained with synthetic media than those containing animal sera or embryo extract, studies were designed to evaluate the contribution of constituents(3). Experiments comparing Eagle's with Mixture 199 had revealed a slightly greater virus yield and a fairly marked enhancement of cytopathogenic changes with the former. Accordingly, the relative effects of glutamine, vitamins and amino acids in Eagle's medium were determined by selective omission of each (Table II). Although no single critical factor was demonstrated, absence of glutamine or vitamins caused more reduction in virus yield.

Effect of pH on growth and stability. It had previously been noted that fall of pH in culture fluids (<6.8) resulted in reduction in viral multiplication and cytopathogenic effects. Adjustment of pH with $NaHCO_3$ solution to 7.6 or above also was followed by lessening of cytopathogenic effects and virus yield remained low. Experiments comparing pH 7.0-7.2 with 7.6-7.8 revealed a 10-100 fold reduction in virus yield and sensitivity to infection at the higher pH. This was observed with either Eagle's or Mixture 199 as maintenance medium. The stability of infectious 2060 in solutions with pH ranging from 1.0 to 11.4 was determined at 4° and

TABLE III. Stability of Infectious 2060 Virus in Different Hydrogen Ion Concentrations and Varying Times and Temperatures of Exposure.

	Conditions and resulting infectivity titers*			
	4°C		35°C	
	30 min.	24 hr	30 min.	24 hr
pH	ID$_{50}$†			
1.0	.0	1.0	.0	.0
5.0	2.5	1.0	2.5	.0
7.1	2.5	—	2.5	1.5
8.8	1.5	1.0	.0	.0
11.4	.0	.0	.0	.0

* Untreated control at pH 7.0 was $10^{2.8}$TCID$_{50}$.

† Reciprocal of logarithm of infectivity titer/0.1 ml.

35°C as shown in Table III. In these experiments the pH of Mixture 199 was adjusted with 1N HCl or NaOH and aliquots sealed in glass ampoules immediately after addition of virus. Following incubation of similarly prepared aliquots for 30 minutes and 24 hours respectively, the pH was adjusted to neutrality and infectivity titers determined. The greatest degree of stability began at neutrality and extended to the acid side. In contrast, even a half hour at 4°C in an alkaline solution caused loss of infectious virus. Increased temperature or time regardless of pH was accompanied by reduction in infectivity titers. Accordingly, 2060 could not be called a very stable virus, and the preference for the acid side of neutrality for maintenance of infectivity demonstrated the need for care in control of hydrogen ion concentration.

Thermal stability. The effect of temperatures of 35-56°C on infectivity was determined at pH 7.2. Aliquots of virus were sealed in glass ampoules and heated for variable periods at the temperatures shown in Fig. 1. Inactivation rate was quite rapid at 56°C for 10 minutes and decreased progressively at lower temperatures. The reduction that occurred at 35°C was appreciable and undoubtedly had a bearing on yield of infectious virus especially when a prolonged growth period was employed. These results classify 2060 as relatively unstable in regards to increased temperature. Methods were not available to determine the stability of non-infectious antigen.

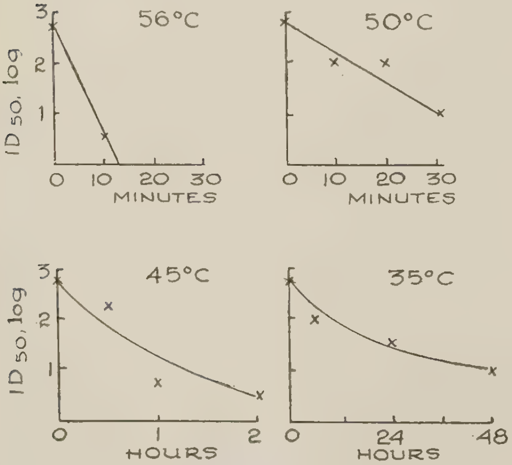

FIG. 1. Rates of reduction of infectivity titers of 2060 by temperatures of 35-56°C.

TABLE IV. Antigenic Relationship between 2060 and JH Viruses by Reciprocal Neutralization with Animal and Human Antisera.

	Neutralization titers‡			
	2060		JH	
Antisera	Acute	Conv.	Acute	Conv.
Animal*				
2060, monkey	11	91 128	<8	8 32
JH, rabbit	<4	<4	4	”
2060, ”	”	128	<4	<8
Human†				
DEF	16	128	8	11
JAB	32	16	8	45
BB	16	128	11	64
WH	64	45	”	11

* Prepared by intrav. inoc. of 10 ml of virus with a titer of $10^{3.5}$TCID$_{50}$/0.1 ml and bled at 14 days. A second inj. was given to the monkey at 14 days and another convalescent blood was obtained at 28 days.

† Representative serum pairs from cases of respiratory disease demonstrating the various types of responses to each virus.

‡ Reciprocal titers of initial dilutions of sera inhibiting $10^{2.5}$TCID$_{50}$.

Antigenic relation to JH virus. Neutralization titers of animal and human antisera were determined simultaneously with 2060 and JH viruses (Table IV) in order to evaluate their degree of antigenic differentiation. Considerable specificity was observed with antisera from animals immunized by a single inoculation of virus, while a second injection increased the neutralization titer to the heterologous virus. Closer relationship was demonstrable with human sera. Four fold or greater increments in neutralization titers with both viruses were found in 32 of 57 human serum pairs. Increases in neutralization titers to only one virus was observed in the remainder.

Prevalence of infection as determined by neutralization titers of human sera. Acute and convalescent sera were collected from young adults with mild, afebrile respiratory illnesses with coryza as the predominant symptom, although mild sore throat and nonproductive cough were also present in some. Group A streptococci were not found by throat cultures and there was no antibody increase by hemagglutination-inhibition with recent influenza A and B strains, except in those recently vaccinated, or by complement-fixation with adenovirus in these individuals. Four fold or greater increments in neutralization titers were observed in 6 of 13 serum pairs obtained during the spring of 1957 and in 10 of 31 obtained during the fall of 1957 in the New Orleans area. Inoculation of garglings from the above persons into monkey kidney cultures in several instances produced cytopathogenic changes characteristic of 2060, but the agents were repeatedly lost on subsequent passage before identification could be accomplished.

Although neutralization titer increments were readily reproducible in separate experiments, titers of individual sera varied considerably. Factors such as degree of development of cytopathogenic changes and effective quantity of infectious virus had a bearing on these results. The significance of neutralization titers in the absence of increases between serum pairs determined simultaneously was, accordingly, subject to question.

Discussion. Although preliminary studies had indicated that 2060 was a very stable agent with relatively slow rate of growth, more detailed observations have shown a considerable degree of instability of the infectious particle in regards to pH and temperature extremes. This demonstrates the need for determinations of characteristics of a virus that include several dimensions simultaneously, including time as a variable rather than a constant. Even rate of growth in tissue culture may be dependent on these properties as well as the type of cell and the constituents of the culture fluid. Serum, later found to be inhibitory to rate of viral multiplication, had previously been included in maintenance media(2,3). On the other hand, the use of roller tubes had produced a greater yield of infectious virus than that derived from stationary cultures. This has been observed by others (8). Such factors influenced results of neutralization titrations and may serve to explain lack of reproducibility of titers in separate experiments. Although titer increments were accurately repeatable, measurements of antigenic differences between JH and 2060 might have been overly sensitive, since lesser amounts of antibody were needed to inhibit smaller quantities of operationally infective virus. Similar considerations reduced the significance of titers of neutralization determined on single human sera and made surveys of antibody levels in various populations of doubtful merit.

Summary. In monkey kidney cultures increments in infectious 2060 could be correlated with the degree of observed cytopathology. The absence of glutamine or vitamins contained in Eagle's medium had some effect in lessening yield of infectious virus, but alterations in pH away from neutrality were more significant in reducing viral multiplication. Infectious 2060 was relatively unstable as evidenced by the inactivation rate at temperatures of 35°C and above and pH below 5.0 and above 7.1. JH virus and 2060 were shown to be related antigenically, but were not identical, as shown by reciprocal neutralization titrations with animal and human antisera. Additional instances of neutralization titer increments in man following symptoms of mild respiratory illness have been presented.

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Received June 2, 1959. P.S.E.B.M., 1959, v102

Production of Experimental Congestive Heart Failure in the Guinea Pig.*
(25262)

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To understand more clearly the basic mechanisms involved in the heart and other organs during the course of chronic congestive heart failure, there is a need further to study congestive heart failure from the viewpoint of bioenergetics(1,2,3). The adaptation of biochemical technics to such a problem requires (a) availability of fresh whole non fibrotic cardiac and other tissue for the various enzymatic and biochemical technics, (b) tissue preparations which are readily reproducible and (c) uniform development of congestive heart failure. These criteria have been met in the technic to be described herein which employs the guinea pig as the experimental animal. The basic principle of the technic is to reduce the size of the arch of the aorta by one-third by means of a ligature. The animals thus treated will develop the syndrome of congestive heart failure(4) within a period of 10 to 12 days with the sequential pattern of changes from enlargement of the left ventricle, to passive venous congestion of the visceral organs. The pathologic picture in the tissues of these animals is similar to that observed in man during the development of chronic congestive heart failure. Tissues from these animals may be subjected to technics suitable for biochemical study.

Methods and materials. An adult male Hartley strain guinea pig of approximately 400 g is anaesthetized by injecting 25 mg of Nembutal intraperitoneally. After the animal is completely anaesthetized, the hair is shaved from the chest and ventral aspect of the neck; the animal is placed in the dorsal recumbent position. A plastic catheter is inserted into the exposed trachea. Oxygen is permitted to flow at the rate of 0.3 liter per minute. A "T" tube is placed between the inflow tract and trachea in order to administer artificial respiration, if necessary, during the operative procedure. An incision is made through the skin, pectoralis major and pectoralis minor muscles in the region of the left 6th intercostal space. The chest wall is opened and self-retaining retractors are placed in the opening. The pericardial sac in the region of the pulmonary arteries is separated. A Deschamp ligature carrier, complete with black braided silk thread size 0 is placed under the aorta as close to its exit as possible. The needle is withdrawn, leaving a double thread encompassing the aorta. After the size of the aorta is ascertained by means of calipers, the ligature surrounding the aorta is closed, so that the aorta remains approximately 33% patent. The chest is closed in 3 stages, (a) thoracic cage is first closed (b) the pectoralis muscles are coaptated, (c) the skin is united. (The collapsed lung is inflated prior to closing the chest wall.) The incision surrounding the trachea is closed, the skin is united and the catheter withdrawn. Fifty mg of ascorbic acid buffered tetracycline (Squibb) is injected intramuscularly into each animal. No special treatment is required post-operatively. Electrocardiograms are taken with the aid of needle electrodes inserted into the animals' limbs and attached to a Sanborn direct writing electrocardiographic instrument.

Results. 1. *Electrocardiographic changes* observed in the guinea pig prior to and after production of congestive heart failure show a shift in axis from the normal to left as well as an increase in voltage of the QRS of 60%. In addition to the increase in voltage of the QRS there is a change in repolarization as evidenced by changes in the S-T segments and T waves. These electrocardiogram changes are indicative not only of an enlarging left ventricle but also of an enlargement of all cardiac chambers.

2. *Pathological changes*† observed in ani-

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* Supported by grant from Nat. Heart Inst. and Cardiac Welfare Fund, N.Y.U. College of Medicine.

† Dr. Magda Rona, Morrisania Hospital and Albert Einstein College of Medicine, kindly offered to stain the tissues for histologic interpretation. Authenticity of congestive heart failure in experimental animals has been attested by Dr. Rona.

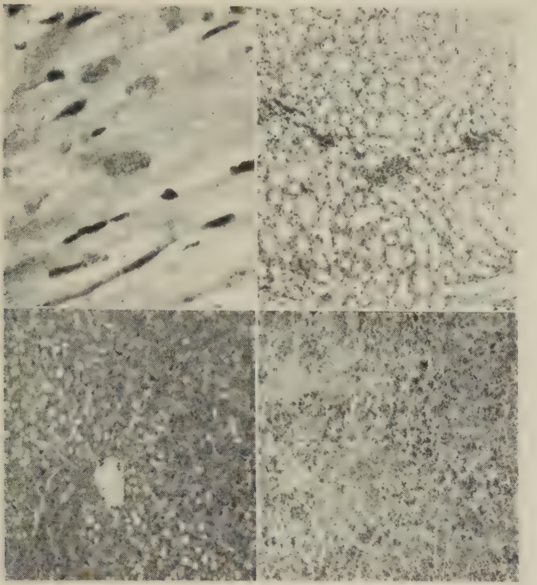

FIG. 1. Microscopic sections of heart (left upper corner), kidney (right upper corner), liver (left lower corner) and spleen (right lower corner) taken from 410 g guinea pig with congestive heart failure produced experimentally. Changes shown are characteristic of chronic passive venous congestion and in the heart, cardiac hypertrophy.

mals with experimentally produced congestive heart failure were typical of the classical changes described in the literature(4). Spleens taken from experimental animals were wider, thicker and heavier than normal controls. (Normal spleen weighs 0.7 g and spleen from congestive heart failure animal weight 2.85 g). Fig. 1 shows 4 microphotographs taken from sections prepared from the heart, liver, spleen and kidney of an experimental animal. Note that the changes observed are typical of congestive heart failure.

Discussion. From a biochemical viewpoint, congestive heart failure may be caused by a failure in (a) energy production, (b) energy transference or (c) energy utilization(2). The more common clinical classification of congestive heart failure suggests "forward" or "backward" failure as the 2 major classifications(2,3,5). In the method described herein, restriction of blood flow through the aorta causes a sequential pattern of changes of enlargement of left ventricle, left atrium, pulmonary edema, enlargement of the right cardiac chambers, and passive venous congestion of liver, spleen and kidneys. The changes described are manifested by (a) electrocardiographic changes, and (b) pathological changes, Fig. 1.

The technic of producing congestive heart failure presented herein offers the possibility of preparing tissue for enzyme and bio-energetic studies which was not as feasible in heart-lung preparations, or in methods previously described for producing chronic congestive heart failure(6,7). The animals in which experimental congestive heart failure is produced may be sacrificed and tissue removed from which slices, homogenates, cell-free suspensions and isolated particles may be obtained. Preliminary work on $Q^{N}_{O_2}$ values and P/O ratios on mitochondrial systems prepared from heart and liver have been obtained(8,9) and similar work is continuing.

Summary and conclusion. An operative technic has been described which causes development of congestive heart failure in the guinea pig within 8 to 12 days. Essentially the technic involves intrathoracic incision and restriction of aortic blood flow by ligature surrounding the aorta. Tissues derived from this preparation may be subjected to biochemical and enzymologic studies because of feasibility of preparing slices, homogenates, cell free suspensions and isolated particles. Preliminary biochemical work has been accomplished with this preparation.

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Received June 5, 1959. P.S.E.B.M., 1959, v102.

Lipemia-Induced Acceleration of Intravascular Clotting II. Effect of Hypoprothrombinemia.* (25263)

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Intravenous injection of a fat emulsion in anesthetized dogs is associated with accelerated clot formation in isolated vein segments (1). The present investigation was concerned with the question of whether such clot acceleration might also occur in dogs in which a hypoprothrombinemic state had been induced.

Material and methods. Mongrel dogs, weighing 13 to 22 kg, were given 25-50 mg of bishydroxycoumarin daily for 3-4 days until their one-stage "prothrombin" time(2) reached levels indicating less than 10% of prothrombin activity.[†] Under Pentobarbital anesthesia, the internal jugular and femoral veins were freed and isolated by clamps, and small segments of vein excised at regular intervals for determination of intravascular clotting time (ICT). This was the interval from moment of clamping veins of one side to removal of segment which contained the first grossly visible fibrin clot(1). Hemostasis during surgical procedure often was quite difficult because of extensive oozing of blood from tissues. Serum was collected before and after fat emulsion[‡] (40 ml) was injected into peripheral vein, for estimation of optical density (at wave length 620 mμ) and of triglycerides(4).

Results. Intravascular clotting time in fasting dogs. In 11 hypoprothrombinemic dogs, ICT was measured in jugular and femoral veins of right side before fat emulsion was injected. These fasting values ranged 35-75 minutes (Table I); 2 control animals and 1 injected with the emulsion showed unduly low ICT's, because vein segments were exhausted before recognizable clot was observed. All dogs except one had plasma prothrombin concentrations less than 1%, the normal range of prothrombin time in dogs being 8-10 seconds. The longest ICT, 75 minutes, was in a dog with prothrombin time of 36 seconds, the longest prothrombin time, more than 100 seconds, was in a dog with ICT of only 55 minutes.

Intravascular clotting time after injection of fat emulsion. In 8 of 10 dogs, ICT determined in (left-sided) veins isolated 2 minutes after injection of fat emulsion was shorter than before injection by 5-35 minutes (Table I). One of the 2 remaining dogs showed the same ICT before and after injection; the lower post-emulsion value recorded for the other dog does not represent a reliable endpoint (see above). Shortening of ICT coincided with marked increase in lactescence, as well as in triglycerides of serum (Table I); the degree of shortening appeared to correspond roughly to the change in serum lactescence.[§] In 2 other dogs, fat emulsion was injected before veins of either side were clamped (upper section, Table I). In one of these dogs, ICT was 10 minutes longer at time of decreasing lipemia, *i.e.*, about 35 minutes after injection, than at peak of lipemia,

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[*] This study supported by grant from Chicago Heart Assn.

[†] This test chiefly reflects deficiency of factor VII (proconvertin) in plasma; after 3-4 days on above dosage, both prothrombin and Stuart factor are usually also depressed but to a lesser and unpredictable degree(3). The term hypoprothrombinemia refers to "prothrombin complex" rather than to prothrombin *per se*.

[‡] This emulsion, containing 15% vegetable oil and 1.2% Soybean Phosphatide, furnished through courtesy of Don Baxter.

[§] Neither parameter of lipemia was sufficiently accurate for analysis of correlation: Hemolysis incidental to collection of blood tended to increase optical density of serum, which was always determined on day of collection; some triglyceride values are probably erroneous because fat emulsion separated from serum after storage at 4°C for one or several days and did not mix well with shaking. Recovery experiments with emulsion added to serum were not very satisfactory. Hence, values for triglycerides and for lactescence listed in Table I will serve to illustrate direction of change of lipemia in each dog but do not lend themselves for precise computation of correlations.

TABLE I. Intravascular Clotting Time (ICT), Serum Triglycerides (TG) and Lactescence (L) before and after I. V. Injection of Fat Emulsion.

	Fasting			2 min. after			35-75 min. after		
Prothrombin time, sec.	ICT, min.	TG, mg/100 ml	L, % T	ICT, min.	TG, mg/100 ml	L, % T	ICT, min.	TG, mg/100 ml	L, % T
45			87	20	84	36	30	54	52
21			63	25	471		25	206	
26	>35	81	66	30	125	31		96	42
22	35		91	35		53			55
40	>40	72	85	20	160	2		128	73
31*	60		82						
45	45	114	79	>35	465	5			
50	65	64	79	55	556	5		225	15
43	65		86	45		10			67
36	75	40	91	65	163	18		105	34
36	55	75	83	35	103	13		72	22
47	65	79	81	30	233	23		134	61
>100	55	83	78	35	211	35		133	42

% T denotes % transmittance.

* Died after additional dose of pentobarbital before fat emulsion was inj.

as determined 2 minutes after injection. In the other dog, ICT was unchanged.

Discussion. ICT values in these severely hypoprothrombinemic dogs tended to be somewhat prolonged‖ when compared with those observed under the same conditions in dogs not pre-treated with bishydroxycoumarin. While untreated dogs usually yielded fasting values of 30-40 minutes(1), the present fasting range was 35-75 minutes and over-all range (for 25 estimations in 13 dogs), 25-75 minutes. All values were within the 20 to 90-minute period considered by Wessler as the normal range; this author had noted normal intravascular clot formation in 17 of 18 hypoprothrombinemic dogs, although slight retardation may have been overlooked(5).

ICT and one-stage prothrombin time showed poor correlation, a perhaps analogous observation that prothrombin time sometimes fails to provide a dependable measure of *in vivo* hypocoagulability or bleeding tendency (2,3). Whether or not ICT might correlate better with deficiency of individual plasma factors, which are coumarin-sensitive, such as prothrombin and Stuart factor, remains to be determined.

In spite of the apparent tendency to retardation of intravascular clot formation observed in coumarin-treated dogs, lipemia induced by administration of fat emulsion resulted in clot acceleration in most instances. While this acceleration might have to do with duration of surgical procedure and some blood loss or exhaustion, observations in the 2 dogs receiving fat emulsion at beginning of experiment seem to disprove this point: ICT was the same or longer on the "second" (left) side. Hence, ICT quite consistently tended to be shorter at height of lipemia, whether this occurred earlier or later in the surgical procedure.

It should be remembered that it may not be over-all "lipemia" but some ingredient of fat emulsion, presumably soybean phosphatide, which exerted the clot-promoting effect (6). Whatever the lipemic agent responsible for this effect, it has one property in common with the (unknown) thrombogenic substance contained in canine serum which, upon rapid intravenous infusion, causes an intravascular clot to form within 2 minutes(7). Neither effect is obviated by marked hypoprothrombinemia and bleeding tendency. This finding suggests that lipemia-induced clot acceleration is independent of that phase in the coagulation process which is reflected in the prothrombin time test, particularly factor VII.

Summary and conclusions. 1) Bishydroxycoumarin-induced hypoprothrombinemia may result in a slight delay of fibrin clot in isolated venous segments of anesthetized dogs. 2) The hemorrhagic state produced by the drug fails to prevent acceleration of intravascular clotting caused by intravenous infusion of fat emulsion. This clot acceleration, therefore, may not operate through the mechanism of prothrombin conversion. 3) Our experiments lend support to the concept that anticoagulation with coumarin-type drugs is not synonymous with antithrombotic effect.

‖ The number of dogs used was considered too small for statistical evaluation.

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Received June 9, 1959. P.S.E.B.M., 1959, v102.

Digital Pre- and Postcapillary Vasoconstriction and Vasodilatation Resembling Pre- and Postglomerular Arteriolar Function in Kidney.* (25264)

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Spontaneous variations in digital vascular volume have been described(1) quantitatively for man. They are generally classified into 5 types—pulse, respiratory, alpha, beta and gamma deflections. *Alpha deflections* have a mean frequency of 7.9/min. (range 2 to 14) and mean magnitude of 14.5 mm^3/5 cc of part (range 0.1 to 81). *Beta deflections* have a mean frequency of 1.3/min. (range 1 to 2) and mean magnitude of 30 mm^3/5 cc of part (range 5 to 60). *Gamma deflections* have a mean frequency of 4/hr (range 1 to 8) and mean magnitude of 150 mm^3/5 cc of part (range 50 to 350). Pulse deflections are superimposed upon alpha, beta and gamma deflections (Fig. 1). With marked vasoconstriction and marked vasodilatation the alpha, beta and gamma deflections are small to almost absent(1,2). The function of these spontaneous volume deflections has been a puzzle. With development of the rheoplethysmographic method for measuring simultaneously time courses of volumes and rates of digital inflow and outflow during a single pulse cycle(2,3), the significance of these spontaneous deflections is becoming apparent. *Theoretic considerations.* Large ascending and descending alpha, beta and gamma deflections are associated with large increases and decreases, respectively, in digital volume.

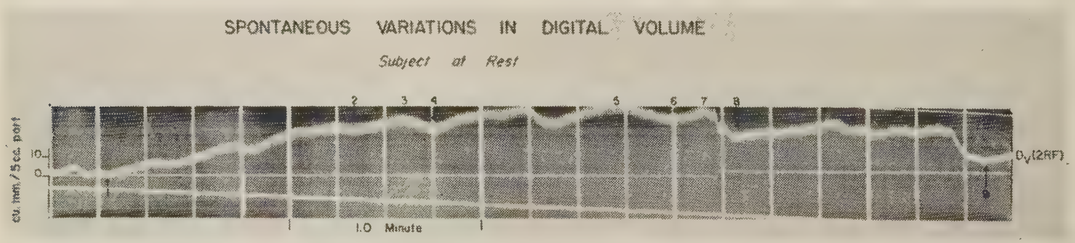

FIG. 1. A segment of a rheoplethysmogram recorded at slow camera speed showing variations in volume, D_V (2RF), of the tip of right second finger (index finger). The pulse waves are superimposed upon alpha deflections (arrows 2 to 3 and 3 to 4, for example) which, in turn, are superimposed upon beta deflection (points 1 to 5 and 5 to 9).

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* Aided by grants from U.S.P.H.S. and the Thibodeaux Fn.

Fig. 1 shows the tip of second right finger (2RF) to undergo marked variations in volume. Arrow 1 of Fig. 1 shows the beginning of an ascending beta deflection of D_v (2RF) which reached its peak at point 5. This ascending beta deflection had several superimposed descending (arrows 3 to 4, for example) and ascending (arrows 2 to 3) alpha deflections which, combined, were associated with a net increase in volume of finger tip of 35 mm^3/5 cc of part in 155 seconds. The descending beta deflection began at point 5 and reached lowest level at arrow 9 in 110 seconds with a net decrease in digital volume of 24 mm^3/5 cc of part. This descending beta deflection also had superimposed ascending (arrow 6 to point 7, for example) and descending (point 7 to arrow 8) alpha deflections. Because of magnitude of volume change during ascending and descending beta deflections, the postcapillary vessels, including digital veins, must have been involved in dilatation and in constriction. It has been found rheoplethysmographically that when an alpha deflection ascends digital inflow increases, and when an alpha deflection descends digital inflow decreases. Therefore, whenever an ascending alpha deflection with its associated increase in rate of digital inflow of blood is located on a descending beta or gamma deflection which reflects a decrease in volume, there must be net dilatation of precapillary digital vessels (arterioles and arteries) with net constriction of postcapillary vessels. Whenever a descending alpha deflection with its associated decrease in digital blood flow is found on an ascending beta or gamma deflection which reflects an increase in digital volume, there must be net precapillary constriction and net postcapillary dilatation.

Results. Fig. 2 and 3 show that rate of inflow (I_R) was greater when alpha deflection was ascending than when descending, indicating precapillary vasodilatation in the former. The trace of volume inflow (I_V) obtained

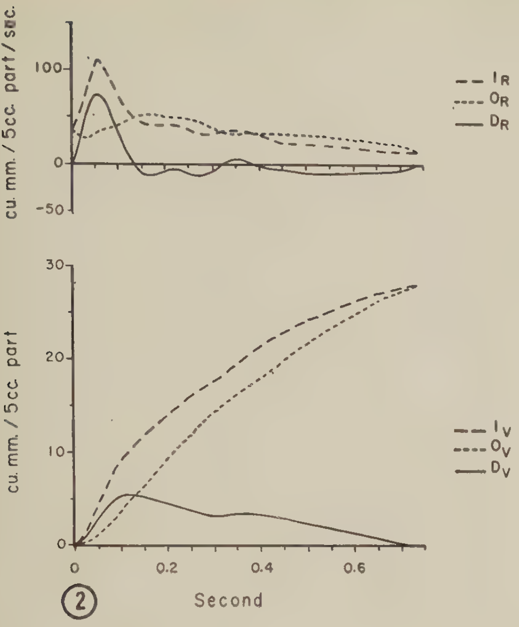

FIG. 2. Rheoplethysmogram of index finger tip (2RF) for a pulse cycle during a descending alpha deflection and an ascending beta deflection. Simultaneous time course curves of rates of digital inflow (I_R) and outflow (O_R) and difference between rates of inflow and outflow (D_R) are shown at top of illustration. Simultaneous time course curves of volume of digital inflow (I_V) and outflow (O_V) and difference between volumes of inflow and outflow (D_V) are shown at bottom of illustration. In this Figure and in Fig. 3, the 3 rate curves are time courses of the first derivatives of the 3 volume-time curves, derived as previously described(2,3). Magnitudes, configurations and temporal relationships of these curves should be compared with those of curves in Fig. 3.

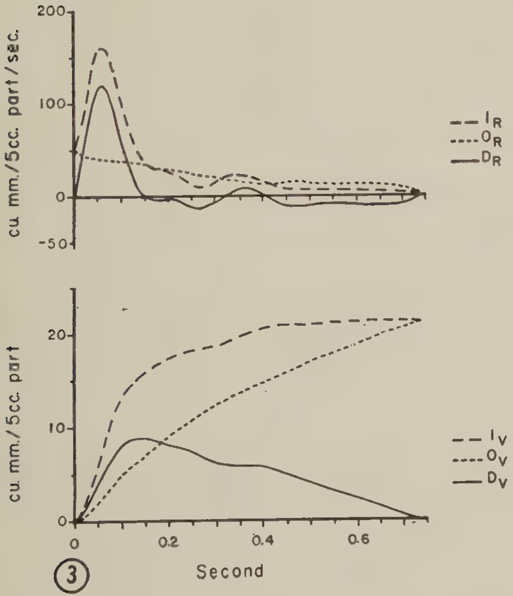

FIG. 3. Rheoplethysmogram for the same digit and subject as in Fig. 2, recorded a few minutes later when alpha deflection was ascending and beta deflection was descending.

when alpha deflection was ascending on a descending beta deflection (Fig. 3) shows a rapid "plateauing off" due to leakage of blood past the collecting cuff as a result of "over-filling" or small capacity of the collecting vessels. The postcapillary collecting vessels accommodated only 18 mm^3 of blood before "over-filling," indicating that these vessels had small capacity and high tone, as would be expected for constricting postcapillary vessels associated with a descending beta deflection. However, when alpha deflection was descending on an ascending beta deflection, these same postcapillary or collecting vessels accommodated 28 mm^3 of blood and still did not "over-fill" (Fig. 2). This situation would be expected in a loose postcapillary vascular system such as would exist if vessels were dilating and, therefore, of relatively large capacity and low tone.

It can be seen from Fig. 1 that the pulse waves increased in volume during ascending beta and alpha deflections, whereas they decreased in volume during descending beta and alpha deflections. Burton(4) and I(2) have shown that, in general, under conditions of these experiments, the rate of digital inflow tends to vary directly with volume of pulse wave. Changes in volume of the pulse wave are further evidence of net changes in calibre of the precapillary vessels.

Discussion. Simultaneous discordant and disproportionate pre- and postcapillary vascular dilatation and constriction, which occur spontaneously at all times in tissues, resemble the functional relationships of afferent and efferent arterioles of the glomerulus of the kidney. These phenomena in tissues must be concerned with regulation of the exchange of fluid and solute between interstitial spaces and plasma. They must also be concerned with maintenance of the circulation of interstitial fluid and lymph. Time courses of lymph flow noted in tissues by McMaster(5) are similar temporally to alpha and beta deflections. This similarity possibly reflects the influence of properly integrated pre- and postcapillary vasodilator and constrictor activities upon capillary filtration of plasma water and solute and capillary reabsorption of water and other substances from the tissues. It may also reflect a source of energy derived from circulating blood which is responsible in part for the flow of lymph fluid in the sacs, capillaries and small vessels of the lymphatics.

The influence of normal physiologic factors, drugs and disease upon temporal relationship and magnitude of pre- and postcapillary vascular constrictor and dilator activity needs study. The relatively thick accumulation of smooth muscle noted in arterioles and small veins could function as pre- and postcapillary "sphincters" in this integrated regulation of the circulation of interstitial fluid and lymph.

Summary. These studies indicate that, in general, alpha deflections reflect net changes in calibre of precapillary vessels and beta and gamma deflections reflect net changes in calibre of postcapillary vessels and that these changes in pre- and postcapillary vessels are probably important in the regulation of peripheral circulation and flow of tissue fluid and lymph.

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Received June 9, 1959. P.S.E.B.M., 1959, v102.

Production of Hormones by Human Anterior Pituitary Cells in Serial Culture. (25265)

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The need to develop methods to supply human-type pituitary hormones has been evident since the demonstration(1-5) of effectiveness in humans of growth hormone from humans, and the realization that specific activity of human-derived hormone in man is due to differences in molecular structure of somatotropins(6-9). Cell culture(10-16), has been applied especially in virus and cancer research, but production of specialized secretion products of cells, such as enzymes or hormones, has not heretofore succeeded(17,18). Explants of placental or pituitary tissue, on the other hand, have produced detectable amounts of hormones in tissue, as contrasted with cell culture(19-21).

Methods. The "starter" tissues (stage I*) comprised portions of 9 anterior pituitary glands, 4 from women, 5 from human fetuses varying in age, and fragments of an acidophilic adenoma from a woman with acromegaly. These tissues were obtained incidental to surgical operations for therapeutic purposes. Immediately after excision, the sterile tissue fragments were separated aseptically from surrounding membranes, and minced and prepared as explant cultures in Eagle's basal medium(11) with serum supplements, either human, equine or bovine. *Serial propagation of heterogeneous cell cultures* (stage II) was undertaken after a sufficient outgrowth from tissue fragments had taken place. Then, by serial subculture, a considerable number of stationary cultures were derived from each starter tissue. Whenever practical at time of transfer, permanently stained coverslip preparations of cells were examined. *Propagation of selected epithelial-like cells in stationary culture* (Stage III): Islands of epithelial-like cells eventually appeared among the fibroblast-like cells of early cultures. These were selected for subculture. With several starter cultures, it was possible to pick out epithelial-like cells from the proliferating explant. *Propagation of selected cell lines in a suspension system* (stage IV) was usually successful with cell concentration of 100,000 to 200,000 cells/ml. In a suspension-type of culture the generation time in most cell lines was about 24 hours. However, 2 lines, S-4374-VAM-I and -II have generation times of about 48-72 hours. Cell counts of all these cultures were maintained with a wide range of from 100,000 to nearly a million cells/ml. Clones have been developed from primary cultures as well as from established hormone-producing cultures. To prepare permanent stained sections of cells of suspension cultures, cubes of gelatin sponge† (5x5x5 mm) were introduced into cultures from 48-72 hours, and were subsequently fixed and stained with acid fuchsin aniline-blue or iron-periodic acid-Schiff(22) methods. *Frozen-storage of cells and their recultivation* (stage V) was carried out as follows: Samples of line 4374-M from both stationary- and suspension cultures were frozen and after several months were re-established, employing methods of Stulberg *et al.*(23) with the following modifications: Suspension cultures in the log phase of growth were used, and on day before freezing, were fed with nutrient medium containing 15% serum. Glycerol was added prior to freezing, and they were not centrifuged. *Assay of hormones in cultures* (stage VI) employed standard endocrine bioassays.‡ *Somatotropin* was tested by measuring width of tibial epiphysis in the female rat hypophysectomized at 30 days of age. Subcutaneous injections were started 12 days after hypophysectomy and were made once daily for 4 days, with autopsy on 5th day. Culture medium alone was used as control. A reference somatotropin (bovine) was used as standard in doses of 25 and 40 μg, 25 μg being smallest dose that caused a clearly positive effect. Each hypophysectomized rat usually tolerated doses of about 200 mg of the acetone-dried preparations, and only about 100 mg of freeze-dried preparations. *Gonadotropin* screening tests were made in 21-day-old female mice, each injected subcutaneously twice daily for 4 days with total dose of 5 or 10 mg of crude preparations, sacrificed at 100 hours then ovaries and uteri were weighed. Additional tests were made in 21-day-old female rats and in 21- and 26-day-old§ hypophysectomized male rats, in which weights of ventral prostate and testis differentiate effects of ICSH and FSH(24). To determine whether observed effects were caused by estrogenic type of substance present in serum of the media, sera and media were tested in castrated immature female rats or in castrated immature female mice, injecting doses larger than or equal to those used in screening tests. *Thyrotropin* was tested in 1-day-old chicks‖ which tolerated doses of 15 mg of acetone-dried harvests. *Corticotropin* was tested by ascorbic acid depletion method in hypophysectomized rats.‖ In harvesting, to prevent enzymatic destruction of hormones, cultures were treated immediately as follows: Some were frozen and aliquots were freshly thawed for each injection; others were frozen, lyophilized and further dried over P_2O_5; and others were precipitated with acetone and precipitates were rewashed with acetone, then ether, dried *in vacuo* and over P_2O_5. The method of choice appeared to be an adjustment of pH to 5 with HCl, followed by acetone precipitation at 92%. Large doses of crude materials had the disadvantage of causing toxic effects and local edema in animals with possibly faulty absorption. To reduce toxicity some samples were extracted(25-27) but the possibility of losses and the cumbersomeness of processing numerous samples led us to rely on tests of crude material in moderate doses.

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* Stages I - V were carried out at Microbiological Assoc.; Stages VI, VII at Organon where Stage IV is being continued.

† Gelfoam, Upjohn.

‡ With assistance of Endocrine Labs., Madison, Wis.

Results. Hormonal assays of heterogeneous cell cultures. Assays indicated gonadotropin, corticotropin and somatotropin activity was present in some cultures in amounts greater than found in sera contained in the media (Table I). *In selected epithelial cell cultures,* gonadotropin and trace amounts of somatotropin were detected (Table II). *In suspension systems,* reached thus far with 15 cell lines, 2 (S4374-M; S4374 VAM-I) showed production of gonadotropin. The gonadotropic action represented combined effects of FSH and ICSH (Tables III to IX). Sera employed in media contained small amounts of uterotropic (estrogen-like) substance (Tables V, VI). Washing of crude powders with acetone and ether did not always eliminate this estrogen-like contaminant. The amount of gonadotropin in harvests such as #051801 was considerably greater than would have been contributed by serum of the media. Gonadotropin was concentrated by ammonium sulfate precipitation followed by ethanol precipitation (Table IX). The amount of FSH present/ml of culture harvest #051801 was approximately that contained in 1 mg of a reference sample of gonadotropin from human menopausal urine (HMG20A). The amount of FSH caused an increase of about 50% in weight of H-rat testes, and this increase we have adopted as our criterion of 1 U of FSH. The amount of ICSH in proportion to FSH in harvest #051801 was roughly the same as as in the menopausal urine preparation, HMG20A, but #051801 had considerably

TABLE I. Hormones Detected in Heterogeneous Cultures.

Culture No.	ACTH	TSH	Gon.	STH
4371-A-23 + A-17	0	0	0	+
4373X-P9	+	0	+	±
4373X-2A	+	0	0	+

TABLE II. Hormones Detected in Selected Cell Cultures.

		No. of positive tests/No. of samples tested	
Cell line	Serum	Gonadotropin	Somatotropin
4373-X	Human	+ 1/1	
4374-M	"	+ 3/4	
" -V	"	+ 1/3	
4375	"	+ 1/4	+ 2/5
"	Calf	+ 2/3	

Tests for thyrotropin and corticotropin were negative in all samples tested.

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§ Rats 26 days old are preferred to 21-day-old rats because more survive hypophysectomy, and yet are sensitive to gonadotropin.

‖ USP XV.

more FSH than did *Gonadophysin*, a sheep-pituitary preparation. Our criterion of a unit of ICSH is an increase of 100% in weight of the ventral prostate of hypophysectomized 26-day-old rats. The apparent lack of ovarian stimulation with the immature female test animal may be explained by quantities of ICSH found, which may act before the ovarian follicles have had an opportunity to become stimulated by FSH.

Identification of cells (stage VII): Cells of early cultures resembled fibroblasts, but in later cultures epithelial-like types also extended in some areas from the edge of explants. In the heterogeneous cell population stage, the fibroblast type which appeared preponderant showed during early transfers remarkable nuclear changes with increased chromatic material, and there were numerous giant cells and imperfect daughter cells. After further transfers, there was less nuclear activity; the nucleoli became more regular in size

TABLE III. Gonadotropin Tests in #051801, #708901, and #709901. Test animals: 21-day-old female mice, 5/group.

Preparation	Dose total/mouse, mg	Body wt, g	Ovarian wt avg, mg	Uterine wt avg, mg	Vagina
#051801*	4.8	13.4	4.4	36.0	5 open
(S-4374-V4-6)	2.4	12.4	4.8	21.0	3 "
Controls	0	"	4.9	11.3	All closed
#708901† (S-4374-M-47-53)	10	13.2	4.2	35.6	3 open
#709901‡ (S-4374-M-45-51)	10	15.1	4.6	52.1	5 "
Controls	0 (saline)	12.6	4.3	12.5	1 "

* 1 ml of suspension culture of S-4374-V4-6 at the time of this harvest contained 300,000 cells and, when acetone-dried, weighed 10.5 mg. It contained 10% human serum.

† 1 ml of suspension culture at time of this harvest contained 350,000 cells and, when acetone-dried, weighed 9.2 mg. It contained 10% of horse serum (lot #101).

‡ 1 ml of suspension culture at time of this harvest contained 285,000 cells and, when acetone-dried, weighed 9.4 mg. It contained 10% of horse serum (lot #101).

TABLE IV. Gonadotropin Test in #051801. Test animals: 21-day-old female rats, 5/group.

Preparation	Dose total/rat, mg	Body wt avg, g	Ovarian wt avg, mg	Ovarian response	Uterine wt avg, mg	Vagina
#051801	20	64	23.7	A few c.l.* in all	104.5	All open
(S4374-V-4-6)	10	63	17.4	A few c.l. in one	83.8	" "
Controls	0	68	19.9	Follicles in all	31.0	All closed

* Corpora lutea.

TABLE V. Estrogen (Uterotropic) Test. Test animals: castrated, 21-day-old female mice, 3/group.

Preparation	Dose total/mouse, mg	Body wt, g	Uterine wt avg, mg	Vagina	Interpretation
#051801 (gonadotropin positive)	4.8	13.8	29.7	All open	Estrogen +
	1.2	14.3	10.9	None "	—
#081801* (gonadotropin negative)	4.8	14.3	31.2	All "	+
	1.2	13.9	14.1	2 "	+
Controls (saline only)	0	14.3	10.5	3 closed 1 open	—

* This acetone-dried harvest of a culture of a cell line which did not produce gonadotropin, as shown by tests in the intact immature rats and mice, is representative of many which contained small amounts of estrogen-like substance. However, there were many such harvests which did not show positive results in these tests.

TABLE VI. Estrogen (Uterotropic) Test. Test animals: castrated, 21-day-old female rats, 4/group.

Preparation	Dose	Uterine wt avg, mg	Vagina	Interpretation
#051801	50 mg equiv. to 4 ml (about) culture	41.1	All closed	Estrogen ±
"	25 mg equiv. to 2.5 ml culture	27.6	*Idem*	0
Medium* (#050801)	30 mg equiv. to 3 ml culture	25.6	"	0
Human serum†	.25 ml‡	30	"	0
" "	.125 ml	35	"	0
No treatment		33	"	0

* Sterile medium containing 10% of human serum was employed for the culture of #051801, and was dried in the same way.

† This serum was employed in the medium #050801 and the culture prior to time of its harvest as #051801.

‡ This dose is approximately the amount contained in 2.5 ml of culture harvest (10% serum).

and were usually 4 in number. When these cultures were incubated for from 6 to 10 days we usually observed in intercellular spaces filaments of pale blue staining material which resembled collagen with acid fuchsin aniline-blue stain. In most cell lines, at intervals in the heterogeneous stage, we noted clusters or whorls of fibroblast-like cells which tightly enclosed a clump of nuclear chromatic material, the details of which were obscure. We determined that these represented nests of epithelial-like cells, for when these clusters were picked out and subcultured, epithelial-like cells proliferated. Islands of epithelial-like cells which appeared could be easily differentiated microscopically from the fibroblast type.

TABLE VII. Differentiation of Type of Gonadotropin in Harvest #051801. Test animals: hypophysectomized, 26-day-old male rats, 4/group.

Preparation	Dose total/rat	Ventral prostate avg, mg	Testes avg, mg
#051801*	30 mg	48.8	611
	15	31.2	511
	7.5	18.8	439
Human menopausal gonadotropin† HMG20A	2 IU (2 mg)	33.4	567
	1 IU (1 mg)	32.5	390
Gonadophysin (Searle)	2 U	42.2	311‡
	1 U	32.5	390§
Controls (untreated)	0	13.1	358

* 1 ml of harvest #051801 yielded 10.5 mg acetone-dry powder, and contained 300,000 cells.

† Kindly supplied by Dr. J. B. Dekanski of Organon Laboratories Ltd., Newhouse by Motherwell, Lanarkshire, Scotland.

‡ Only 2 animals survived, and 1 of these had unduly small testes.

§ 1 rat had unduly large testes (504 mg) although no remnant of the pituitary could be found. Only 3 rats survived.

TABLE VIII. Differentiation of Type of Gonadotropin in Harvests #708901 and #709901. Test animals: hypophysectomized, 26-day-old male rats, 3/group.

Preparation	Dose total/rat	Ventral prostate avg, mg	Testes avg, mg
#708901	50 mg	19.4	411
	37.5	36.7	324
#709901	50	26.5	390
	37.5	34.4	574
Medium*	5 ml (equiv. to about 50 mg #708901)	14.7	317
"	3 ml	9	265
Serum #101 (horse)	.5 (equiv. to 5 ml medium)	12.1	301
Idem	.3	15.3	266
Controls (saline)		11.7	270

* This medium is a sample of that employed for the cultures that yielded harvests #708901 and #709901 and they all contained serum #101 (equine) in the amount of 10%.
1 ml of #708901 = 9.4 mg.
1 " " #709901 = 9.2 " .

The cell which appears to be typical of the gonadotropin-secreting line (S4374-M), when stained by the iron-PAS method, contains in the cytoplasm oval or round masses which appear pink in bright field illumination and peacock blue in phase. These cells growing on glass frequently extend pseudopods, and they do not remain as firmly attached as do many other types of cells. Under phase microscopy, the unstained living cells of the gonadotropin-producing line of suspension cultures have numerous oval or round highly translucent masses irregularly placed in their cytoplasm which also contains clusters of fine dark granules best visualized by a green or blue filter.

TABLE IX. Partial Purification of Gonadotropin. Test animals: hypophysectomized, 26-day-old male rats, 3/group.

Preparation	Dose total/rat, μg	Ventral prostate avg, mg	Testes avg, mg
#731B901*	400	31.7	473
	120	17.7	383
	75	20.5	348
	0	11.6	280

* #731B901 was prepared as follows: Harvest #051801 (Table VIII) was subjected to ammonium sulfate precipitation, and portion which precipitated between 33% and 75% saturation was freed of ammonium sulfate by dialysis. This solution, clarified, was precipitated with ethanol at 80% concentration. Precipitate was taken up in 0.5 M ammonium acetate solution at pH 5.0 and ethanol was added to a concentration of 40%. Precipitate was removed and ethanol added to 80% concentration. This precipitate was freed of ammonium acetate by dialysis, precipitated with ethanol at 80% concentration, washed with ethanol and ether and dried over P_2O_5. This was sample #731B901.

The nucleus is a clear area in which the nucleoli are seen as dark masses, best visualized with a red filter.

A chromosome count was made with cells of line S4374-M-32B when serially cultured for nearly 8 months. This particular line was started on 7/3/58 with a fragment of human fetal pituitary gland, and was cultivated for 6 weeks in the heterogeneous cell stage, and for 20 weeks in the selected cell stage; thereafter it was in suspension culture for 5 weeks, at which time a division of the culture was submitted for a chromosome count. Bio-assay at this time showed that gonadotropin production was continuing. The count on the fixed, squashed preparation after colchicine treatment(28) was:

Chromosome No.	No. cells	Chromosome No.	No. cells
76	2	79	11
77	1	80	5
78	3	81	3
		Total	25

This was interpreted as unmistakably primate. The cell strain is heteroploid and the presence of a few telocentric or acrocentric chromosomes indicates that chromosome rearrangement has occurred in the strain's history.¶ This chromosome count was repeated 3 months later (after frozen storage and recultivation), with the finding of a high peak at 79-80 chromosomes per cell.

Summary. Serial cultivation of cells obtained from the human pituitary is described. Somatotropin, corticotropin, and gonadotropin have been detected in these cultures. Two of the cell lines have produced gonadotropin, both FSH and ICSH, when in suspension culture, and one, S4374-M, has been frozen and recultivated in both stationary- and suspension types of culture with media containing 10% of human, equine or bovine serum, with resumption of production of hormone. The gonadotropin-producing cells have the microscopical appearance of a chromophobe type of pituitary cell and have a characteristic appearance when stained by the iron-PAS method. These cells are heteroploid with a chromosome count in narrow range with modality at 79.

¶ We are indebted to Dr. T. C. Hsu, Univ. of Texas, Houston, Texas, for this interpretation of his chromosome counts.

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Received June 22, 1959. P.S.E.B.M., 1959, v102.

Mechanism of Tumor Inhibition by Estrogen.*† (25266)

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It has been shown that estrogen enhances the development of immunity against transplantable tumors in mice(1) and the postulate has been advanced that inhibition of growth of transplantable tumors during pregnancy might be due to hyperestrogenemia(2). The administration of estrogen also increases production of endometrial secretions (uterone)(3,4) and enhances biosynthesis in the endometrium(5). Since endometrial secretions were shown to inhibit growth of transplanted tumors in mice under certain conditions(6), it was logical to hypothesize that some of the tumor-inhibiting effects observed during induced or physiological hyperestrogenemia might be mediated through the uterus (6). The present paper deals with a study to determine the possible role of the uterus in tumor inhibition by estrogens.

Materials and methods. Forty immature AKD2F$_1$ (AKS x DBA/2) female mice, obtained from the Jackson Memorial Laboratory, housed in air-conditioned animal rooms throughout the experiment, were fed Purina Laboratory Chow Checkers and tap water *ad libitum*. The animals were divided into 4 groups of 10 individuals, and surgery was performed on all 4 groups (under nembutal anesthesia) according to the protocol shown in Table I. At operation, estradiol benzoate pellets of known weight were implanted subcutaneously in Groups II and IV. All animals were given achromycin in their drinking water for 5 days following surgery, and on the eleventh postoperative day tumor transplants were made. Sarcoma 1 cells from an AKS female host (obtained from Jackson Memorial Lab.) were suspended in chilled Ringer's solution according to the cytosieve method of Snell(7) and adjusted to a high cell concentration (17%) as measured by means of a Wintrobe hematocrit tube. All animals were injected subcutaneously with 0.2 cc of the same tumor suspension. The order of the injections was arranged so that an identical num-

TABLE I.

Groups of 10 mice	Surgical condition	Treatment
I	Intact (sham operation)	
II	*Idem*	Subcut. estradiol benzoate pellet
III	Hysterectomy & castration	
IV	*Idem*	*Idem*

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* Supported in part by grant from Nat. Cancer Inst., U. S. Public Health Service, and Sciences and Arts Fn., Dedham, Mass.

† Achromycin was supplied through courtesy of Lederle Labs. Division, Am. Cyanamid Co., N. Y.

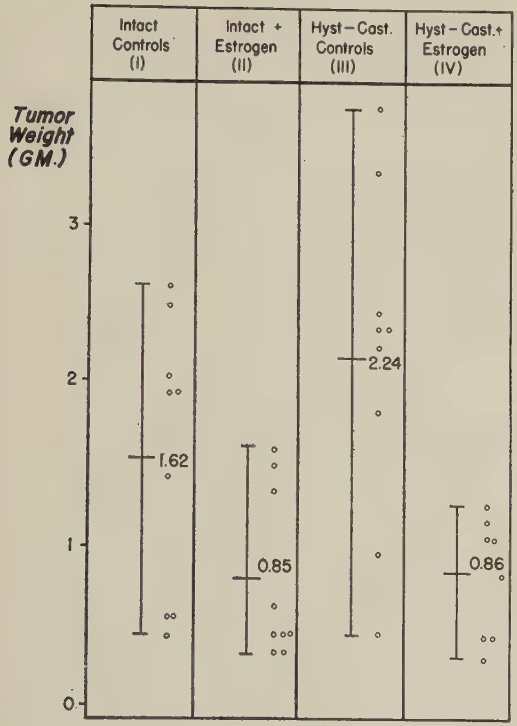

FIG. 1. Weights of tumors (avg and range) in estrogen-treated animals and controls.

ber of animals in each group received cells of similar suspension age. The animals were sacrificed by cervical dislocation on the 10th day following injection of the tumor. The tumors were dissected free of surrounding tissue, weighed, and placed in Bouin's fixative for sectioning and staining. The uteri (where present) were also excised and prepared for histological examination. The estrogen absorbed was determined by subtracting the dry weight of the pellets recovered at the end of the experiment from the initial pellet weight. This dose averaged 0.23 mg of estradiol benzoate per day per mouse.

Results. The results in terms of tumor weights are shown in Fig. 1. Body weights of all animals increased on the average during the experiment, and there were no significant differences between the groups.

In estrogen-treated animals, regardless of presence or absence of the uterus, tumor weights were significantly smaller than in untreated controls.

The reduction of tumor weights in estrogen-treated hysterectomized castrates (Group IV) was statistically significant when compared with their controls (Group III) (p. 0.005), as was the reduction in estrogen-treated, intact animals (Group II compared with Group I) (p. 0.025).

Tumors from both estrogen-treated and untreated animals showed variable amounts of necrosis and hemorrhage. Tumors from the estrogen-treated animals appeared to have thicker fibrous capsules and more active stroma than did those from animals not receiving estrogen.

The tumor growth in untreated, hysterectomized castrates (Group III) seemed to be greater than in untreated, intact animals (Group I), although this difference was not statistically significant (p. 0.1).

The uteri of the estrogen-treated intact animals showed evidence of marked estrogenic effects, with pronounced glandular changes. Immature uteri were found in the intact, untreated animals.

Discussion. It appears that inhibition by estradiol benzoate of transplanted Sarcoma 1 in AKD2F_1 hybrid female mice is independent of the uterus, since this inhibition was exactly equal in hysterectomized castrated mice and in intact animals. It would therefore seem that the tumor-inhibiting effect of pregnancy(2) and of endometrial secretions (6) are due to different mechanisms. Since uterone is devoid of estrogenic effects (unpublished), its tumor-inhibiting mechanism remains to be elucidated.

Summary. Immature AKD2F_1 mice were examined for possible estrogenic inhibition of Sarcoma 1. Large doses of estradiol benzoate inhibited growth of tumor in intact animals and in hysterectomized castrates. It was concluded that this estrogenic effect can be achieved in absence of uterus, and that the tumor-inhibitory properties of endometrial secretions act by a mechanism different from that of estrogen.

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Received June 29, 1959. P.S.E.B.M., 1959, v102.

Comparison of Tolbutamide and Insulin Pre-Treatment Prior to Alloxan Induced Diabetes. (25267)

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Introduction of sulfonylurea compounds as oral hypoglycemic agents in therapy of mild diabetes has stimulated numerous studies to determine the mechanism of action of these drugs. Comprehensive symposia of these works have been published(1-4). Many investigators interpreted their findings as demonstrating that these drugs stimulate release of insulin by the pancreas and produce their hypoglycemic effect by increasing amount of available insulin. Insulin and glucose partially protect starved animals from alloxan diabetes(5) while starvation potentiates diabetogenic activity(6). This study was undertaken to compare the effect of insulin pretreatment with tolbutamide pre-treatment on the diabetogenic potency of alloxan in rats.

Methods. Sprague-Dawley virgin female rats, weighing between 130-180 g, were used. In any one series the average weight of individual groups within that series did not vary by more than 5 g. The diabetogenic agent (alloxan-monohydrate) was injected subcutaneously on the basis of its anhydrous weight at a dose of either 150 or 175 mg/kg of body weight. Glucose was given either orally *ad lib* or injected intraperitoneally at a dose of 5 g/kg of body weight in the form of a 20% aqueous solution. Tolbutamide* was injected subcutaneously in all groups at a dose level of 200 mg/kg at a site on the opposite side of the body from that of the alloxan injection. Commercial crystalline zinc insulin was diluted in saline and injected subcutaneously at a dose of 1 unit/kg. A modified Somogyi-Nelson technic(7,8) was used for blood sugar determinations. Blood was taken from the tail of non-fasting animals from 24 hr through 6 days after administration of alloxan. Normal blood sugar range was found to be between 60 and 120 mg%, using the above technic. Microscopic sections were prepared of the pancreas, kidney and liver from animals in each group studied. All tissues were stained with hematoxylin-eosin (Harris). In addition the pancreatic tissues were stained according to Gomori for beta granules. For liver glycogen studies the animals were sacrificed by breaking the neck. Duplicate liver samples were rapidly removed, weighed and homogenized in 30% KOH. Glycogen determinations were done by the method of Hawk, Oser and Summerson(9). *Series I.* Following fasting period of 48 hr 15 animals were divided into 3 equal groups and additionally pre-treated as follows: 1) no further treatment; 2) glucose oral *ad lib* 6 hr; 3) tolbutamide 200 mg/kg subcutaneously followed by glucose oral *ad lib* 6 hr. The above pretreatment was followed by subcutaneous injection of 175 mg/kg of alloxan. *Series II.* After 48 hr fasting period another series of animals were divided into 4 groups and further pre-treated as follows: 1) no additional treatment; 2) glucose oral *ad lib* 3 hr; 3) tolbutamide 200 mg/kg followed by glucose oral *ad lib* 3 hr; 4) insulin 1 unit/kg followed by glucose oral *ad lib* 3 hr. Alloxan at a dose level of 150 mg/kg was injected subcutaneously into all 4 groups following the above pre-treatment. *Series III.* Thirty animals were fasted 48 hr before intraperitoneal injection of 5 g/kg of glucose. These rats were then divided into 3 equal groups and treated

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* Kindly supplied by Dr. C. J. O'Donovan of the Upjohn Company, Kalamazoo, Mich.

TABLE I. Effect of Various Pre-treatments on the Survival of Alloxan Treated Rats.

Series 1—Alloxan 175 mg/kg

Pre-treatment	Survived	Died	P values†	
	Avg blood sugar (mg %) 48 hr post-alloxan		*vs* 1	*vs* 2
Starvation 48 hr	0	5 743 (2)*		
Idem + glucose oral *ad lib* 6 hr pre-alloxan	3 664	2 1200	.04	
Idem and tolbutamide 200 mg/kg 6 hr pre-alloxan	0	5 730 (3)	1.00	.04

Series 2—Alloxan 150 mg/kg

Pre-treatment	Survived	Died	P values†		
	Avg blood sugar (mg %) 72 hr post-alloxan		*vs* 1	*vs* 2	*vs* 3
Starvation 48 hr	5 491 (2)*	5 415 (3)			
Idem + glucose oral *ad lib* 3 hr pre-alloxan	5 398 (4)	4 356 (3)	.83		
Idem and insulin 1 u/kg 3 hr pre-alloxan	6 180 (1)	4 396 (4)	.65	.90	
Starvation 48 hr + glucose oral *ad lib* and tolbutamide 200 mg/kg 3 hr pre-alloxan	0	8 337 (5)	.02	.01	.01

Series 3—Alloxan 150 mg/kg

Pre-treatment	Survived	Died	P values†	
	Avg blood sugar (mg %) 48 hr post-alloxan		*vs* 1	*vs* 2
Starvation 48 hr + glucose 5 g/kg I.P. 3 hr pre-alloxan	10 459	0		
Idem and insulin 1 u/kg 3 hr pre-alloxan	10 397	0	1.00	
Starvation 48 hr + glucose 5 g/kg I.P. and tolbutamide 200 mg/kg 3 hr pre-alloxan	3 466	7 752	.005	.005

* No. of animals on which blood sugars were obtained; all other averages represent total number. † Chi square probability.

as follows: 1) no further treatment; 2) insulin, 1 unit/kg; 3) tolbutamide, 200 mg/kg 3 hr after the above pre-treatment, 150 mg/kg of alloxan was injected subcutaneously. *Series IV.* Thirty animals were fasted 48 hr, divided into 3 equal groups and further treated as in Series 3. Two additional rats from each of the above groups, as well as 2 starved and 2 non-treated animals were used for liver glycogen determinations.

Results. The results in Series 1 (Table I) indicate that addition of tolbutamide altered the protective effect of glucose against the lethal action of alloxan. The difference between these groups was found to be significant ($p = 0.04$). In Series 2 (Table I) mortality rate of glucose-tolbutamide group was significantly greater than any of the other 3 groups ($p = 0.02$). Results in Series 3 (Table I) show no difference between glucose and glucose plus insulin treated groups. The group receiving glucose plus tolbutamide had an increased mortality rate significantly higher than either the glucose or insulin treated group, ($p = 0.005$).

In Table I blood glucose values are shown for each of the 3 series of experiments. All animals studied developed moderate to severe diabetes regardless of the treatment. All animals which died had blood sugar levels as high, if not higher, than those which survived. It was also observed that rats having levels in excess of 700 mg% rarely lived. Microscopic sections of the pancreas showed complete degranulation in all animals which died. These findings appear to indicate that death

TABLE II. Summation of Comparable Groups in Series 1, 2 and 3.

Pre-treatment	No. of animals	Survived	Died	P values* vs total	vs 1	vs 2	vs 3
Starvation 48 hr	15	5	10	.25			
Idem + glucose oral *ad lib* or I.P.	29	20	9	.005	.02		
Idem and insulin	20	16	4	.005	.005	.45	
Starvation 48 hr + glucose oral *ad lib* or I.P. and tolbutamide	28	3	25	.005	.07	.005	.005
Total	92	44	48				

* Chi square probability.

was a result of diabetes.

A summation of comparable groups in Table I is shown in Table II. In the fasted and the tolbutamide pre-treated groups most of the animals died. Conversely in the glucose and the insulin pre-treated groups over 70% of the animals lived. The fasted and/or the tolbutamide group differed significantly from either the glucose or insulin treated group.

Series 4 was treated the same as Series 3 but failed to show severe diabetes and no deaths occurred. There was no apparent explanation for the difference in response between the 2 series. A comparison of blood sugar levels 5 days following alloxan injection is shown in Table III. The frequency of hyperglycemia is not significantly different between the glucose and the glucose plus insulin pre-treated groups. The increased incidence of hyperglycemia in the tolbutamide pre-treated group is most likely different from the insulin group ($p = 0.075$).

The liver glycogen studies show that the animals receiving I.P. glucose following starvation attain a normal level of liver glycogen. The starved animals had approximately 10% of the normal value. The insulin or tolbutamide pre-treatment resulted in liver glycogen approximately 60% of the non-treated group.

Discussion. We found a consistently greater mortality or hyperglycemia in starved or glucose plus tolbutamide pre-treated animals than in glucose or glucose plus insulin pre-treated groups. Regardless of route of glucose administration these differences remained unaltered. Because the insulin and tolbutamide pre-treated animals had similar liver glycogen content it may be assumed that the differences in alloxan response are not related to liver glycogen. If tolbutamide stimulates and insulin suppresses pancreatic activity, it might appear that the extent of activity at time of alloxan injection would explain the differences. However, glucose which stimulates pancreatic release of insulin has an alloxan inhibitory effect similar to insulin and opposite from tolbutamide. Starvation and tolbutamide both potentiate alloxan. Assuming pancreatic stimulation by tolbutamide and no stimulation by starvation, the secretory activity again cannot be correlated with the diabetogenic activity of alloxan. Regardless of the mechanism, when animals are pre-

TABLE III. Effect of Various Pre-treatments on Blood Sugar and Liver Glycogen Levels in Alloxan Treated Rats. Series IV.

Pre-treatment	Alloxan, mg/kg	Liver glycogen, %	Blood sugar (mg %) 5 days post-alloxan		P values*	
			Above 120	Below 120	vs 3	vs 4
Normal (non-treated)		2.39				
Starvation 48 hr		.22				
Idem + glucose 5 g/kg I.P. 3 hr pre-alloxan	150	2.32	4	6		
Idem and insulin 1 u/kg 3 hr pre-alloxan	150	1.46	3	7	.65	
Starvation 48 hr + glucose 5 g/kg I.P. and tolbutamide 200 mg/kg 3 hr pre-alloxan	150	1.54	7	3	.20	.07

* Chi square probability.

treated prior to alloxan induced diabetes, tolbutamide has an effect different from insulin.

The present findings may be due to a toxic effect and not related to pancreatic function; however daily doses in rats as high as 500 mg/kg of tolbutamide for 9 months have shown no histological changes in the internal organs except the pancreas(10).

Summary. 1) Starvation or glucose plus tolbutamide potentiates the diabetogenic activity of alloxan with increased mortality. 2) Glucose alone or glucose plus insulin protects against alloxan diabetes. 3) Different results obtained with insulin or tolbutamide cannot be explained on the basis of liver glycogen content, blood glucose, or theoretical status of pancreatic activity at time of alloxan injection.

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Received June 29, 1959. P.S.E.B.M., 1959, v102.

Transfer of Acquired Immunological Tolerance of Skin Homografts in Mice Joined in Parabiosis.* (25268)

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Acquired immunological tolerance has been produced in several species of animals(1-4). As originally defined, the phenomenon is produced by exposing animals to antigens during period of immunological immaturity. Immunological tolerance to subsequent tissue or organ homotransplantation has usually been achieved by injection of the prospective recipient with viable hematopoietic cells from the prospective donor. In most instances spleen cells have been used, injected either subcutaneously or intraperitoneally during embryonic life or intravenously in the immediate neonatal period. Hasek's technic(4) of embryonal parabiosis has also been effective in production of immunological tolerance. Tissues successfully homotransplanted following establishment of immunological tolerance have been skin in mice(1,2,5), skin in fowl(6), skin in rats(3,7), skin in dogs(8), ovaries(9), adrenals(10) and hypophyseal glands in mice (11). Moreover, mice have been made tolerant to homologous adenocarcinoma by subcutaneous injection of tumor cells in the neonatal period or by injection of viable homologous spleen cells from the prospective donor strain prior to birth, followed later by graft of the tumor from the donor strain (12). To date, then, all examples of true immunological tolerance have been achieved by exposure to donor strain antigen prior to delivery or in the immediate neonatal period. Recently, however, we observed that weanling Z(C3H) mice, injected with viable spleen cells taken from (Z x Ce) F_1 hybrid mice, became tolerant and accepted skin grafts from (Z x Ce) F_1 hybrid donors(13). Similarly, female mice of the A and C57 Bl (subline 1) strains were made tolerant of male skin isografts, either by intravenous injection of male spleen cells in older animals or by establishing a parabiotic union of adult females with isologous males(14). Finally, using the technic of parabiotic union, it has also been demonstrated that adult mice growing homolo-

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* Aided by grants from Minnesota Division of Am. Cancer Soc., U.S.P.H.S., and the Am. Cancer Society.

gous mammary adenocarcinomas following homotransplantation of tumor immediately after birth appeared to transfer their tolerant state to normal isologous mice of the same strain(unpbl). In the latter experiment, the previously unconditioned parabiont lost its ability to reject the homologous tumor and, like its partner which had been made tolerant by injection of tumor in the neonatal period, permitted progressive growth of the tumor homotransplant. On the basis of these observations, experiments have been performed to ascertain whether or not tolerance of homologous skin grafts, induced in mice by neonatal injection of spleen cells, can be transferred by parabiosis to normal, unconditioned adult animals of the same strain. This communication reports experiments in which acquired immunological tolerance of Ce skin was apparently transferred from tolerant Z(C3H) mice to previously unconditioned Z(C3H) mice by placing the latter in parabiosis with the former.

Material and methods. Highly inbred mice of the Z† and Ce strains were used. Tolerance was induced in mice of the Z strain by intravenous administration during neonatal period of viable spleen cells taken from adult donors of the Ce strain. The technic for intravenous injection in newborn mice and for preparation of spleen cells has been described (5). Each Z newborn received from 3 to 5 million viable Ce spleen cells. Cell viability was determined by the Schrek method(15). Injected mice were used approximately 2 months after weaning. In one set of experiments (Group I), pretreated Z mice received a full-thickness skin homograft taken from adult Ce donors, followed one month later by establishment of parabiosis with normal, non-treated isologous mice. Parabiotic pairs were kept together for 15 days, at which time the normal partner of each pair received a full-thickness skin graft taken from a Ce donor. In another group, (Group II) adult Z mice, which also had been treated at birth with Ce spleen cells but had not been tested for tolerance of Ce skin, were joined in parabiosis with normal, non-treated isologous animals. In this group, after both animals had been in parabiosis for 15 days, the union was surgically discontinued, and 8 days later both members received skin grafts taken from Ce homologous donors. Finally, a group of controls consisting of normal, non-treated mice of the Z stock, joined in parabiosis with other normal mice of the same strain, were studied. After 30 days union, each member was grafted with a piece of skin taken from Ce donors. In most instances, mice were kept under observation for at least 3 months following skin grafts, and in some groups the follow-up period was extended to one year. Parabiosis was of the celomic type(16,17), briefly described as follows: After nembutal anesthesia, both animals were placed parallel to each other, and lateral incisions were made including skin and muscle extending from base of ear to proximal insertion of the femur. To achieve confluence of celomic cavities, the incision was extended into the peritoneal cavity from lower edge of last rib to the iliac crest. The peritonea of the 2 animals were then sutured together with surgical 5-0 catgut. Both skin and muscular layers were joined with skin clips. No bandages or dressings of any kind were used. Parabiotic pairs were housed individually in plastic cages with free access to Purina Fox Chow and tap water.

Results are summarized in Table I. In the first experiment, (Group I in the Table) the previously unconditioned Z parabionts failed to reject the Ce skin homotransplant in a high percentage of cases (Fig. 1).

In the second experiment, (Group II), both animals conditioned at birth by intravenous injection of viable Ce spleen cells and those conditioned only by having been in parabiosis with the pretreated animals, tolerated the homologous skin graft in a high percentage of cases.

In contradistinction to these results were those obtained in the control mice, (Group III). In this experiment, consisting of 12 adult, normal Z mice placed in parabiosis with 12 additional normal Z mice all rejected the Ce skin homotransplant at the usual time for rejection of skin grafts (Fig. 2).

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† Z is used to simplify designation of C3H mice originally obtained in 1956 from Dr. John J. Bittner's laboratory.

TABLE I. Transfer of Acquired Immunological Tolerance of Skin Homografts by Parabiosis.

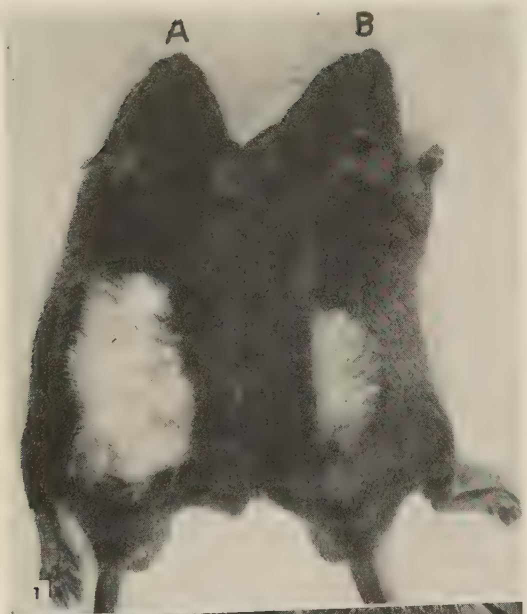
Group	Parabiosis	Days in parabiosis*	No. of pretreated parabionts accepting Ce skin grafts†	No. of non-pretreated parabionts accepting Ce skin grafts†
I	C3H tolerant of Ce skin × normal C3H	15	11/11 (100%)	8/11 (73%)‡
II	C3H pretreated with Ce spleen × normal C3H	15	14/17 (82%)§	10/14 (71%)§
III	Normal C3H × normal C3H	30		0/24 (0%)

* Time in parabiosis prior to transplantation.
† Numerator indicates No. of successful skin grafts and denominator No. grafts attempted.
‡ Transplantation of Ce skin performed while animals were still maintained in parabiosis.
§ Ce skin graft was performed 8 days after separation of parabiotic union.

Discussion. These results demonstrate that failure to reject Ce skin homotransplants is a regular characteristic of Z strain mice which have been placed in parabiosis with Z mice previously made tolerant of Ce tissues by injection of Ce spleen cells at birth. In all instances where successful homotransplantation has been recorded, the graft was maintained in excellent condition during entire period of observation ranging from 3 months to one year. Similar results were obtained when grafting of Ce skin was carried out in the "normal" parabiont 8 days following discontinuation of a parabiotic union with a tolerant mouse which had lasted 15 days. These observations appear to indicate that integrity of the parabiotic union is not essential to success of subsequent homotransplantation. These findings support and extend our previous observations, indicating that failure to reject homologous adenocarcinoma can be transferred to otherwise resistant isologous mice by placing them in parabiosis with an animal tolerant to the tumor.

It is difficult to explain these findings in the light of our present understanding of transplantation immunity and immunological tolerance. Although other bases might be operating, it seems likely that the parabiotic union in some way permitted transfer of the tolerant state from animals in which tolerance has been produced in the conventional manner to the normal partner. This possible interpretation is supported by 2 additional observations. The first is(18) that tolerant mice possess in their spleens all antigens capable upon injection into newborn isologous animals of conferring tolerance to the original donor strain. Since tolerance transferred in this manner persists indefinitely, it may be presumed that antigens involved are present as intact cells derived from the original donor.

Secondly, we observed that in certain well defined and perhaps limited systems, tolerance need not be produced in neonates but may be achieved in much older animals, either by intravenous injection of spleen cells or by establishment of a parabiotic union(13,14). It seems of real significance that in experiments herein reported failure of the transplantation reaction, and very likely immunological tolerance, have also been produced by presumed exposure to foreign antigen in animals well beyond the neonatal period. It seems possible that during the period of parabiotic union between Z mice tolerant of Ce tissue and normal Z mice a continuous transfer of Ce antigens has occurred, and that the dosage obtained and perhaps the constant presence of antigens involved have been sufficient to abrogate the immunological resistance of the normal Z strain parabiont to the homologous Ce skin homotransplant. It is significant that the apparent immunological unresponsiveness seems to be permanent and consequently indistinguishable from that induced by injection of Ce spleen cells into Z mice during the neonatal period(5). The best possible explanation for these observations appears to be that intact Ce cells have been transferred across the parabiotic union from the tolerant animal to its partner, thus inducing tolerance in the previously normal host. If this be the case, it is difficult to understand why intravenous injection of Ce spleen cells in adult Z recipients does not produce tolerance but rather im-



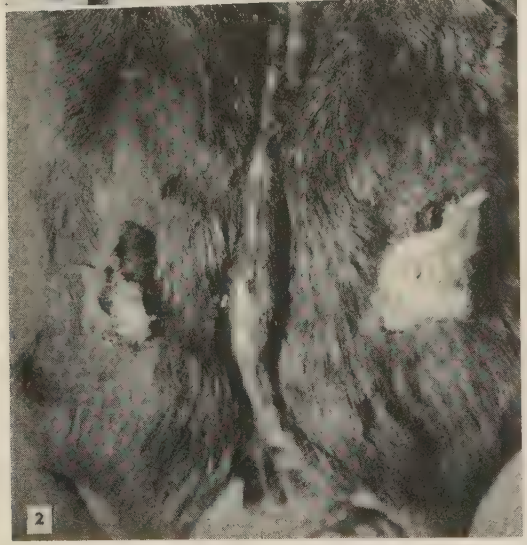


FIG. 1. Transfer of tolerance of Ce homologous skin graft in Z (C3H) mice joined in parabiosis. In mouse A tolerance was induced by neonatal inj. of Ce spleen cells. Mouse B, non-treated at birth shows tolerance of Ce skin after being in parabiosis with mouse A for 15 days.

FIG. 2. Parabiosis between 2 normal Z (C3H) mice during 30 days and subsequently grafted with Ce homologous skin. Note sloughing of Ce graft in both members of pair.

munity. Perhaps, as might be inferred from Egdahl and Hume's(19) experience with cross circulation in dogs and Kamrin's(20) experience with parabiosis in rats, the parabiotic state permits maintenance of a homotransplant of multipotent Ce cells for a sufficient period to permit them to induce a tolerant state which could not be induced by a single intravenous injection of spleen cells at this age. Whatever the mechanism responsible for this phenomenon might be, the observations reported herein provoke additional reflection on possible relationships between immunological tolerance, immunological paralysis(21), and other forms of specific immunological unresponsiveness(22-25).

Experiments to ascertain the essential duration of the parabiotic union, attempts to extend these observations to other inbred strains of mice, and transfer studies to determine whether Ce cells are, indeed, present in spleens of adult animals made tolerant in this manner, are in progress.

Summary. 1. Normal, adult Z(C3H) mice, connected in parabiotic union with Z(C3H) mice tolerant of Ce tissue, fail to reject homografts of Ce skin. 2. Lack of immunological responsiveness to Ce tissues persists following disruption of parabiotic union. 3. The possibility that failure of transplantation reaction is due to transfer of immunological tolerance to previously unconditioned adult recipient is presented. 4. The implications of these observations are discussed.

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Received July 1, 1959. P.S.E.B.M., 1959, v102.

Effect of Thyroxine, Thyrotrophic and Somatotrophic Hormones on Skin of Dwarf Mice.* (25269)

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It is well known that thyroid hormone affects the composition of connective tissue. Histological studies have shown that the metachromatic mucopolysaccharide-containing ground substance decreases and mast cells decrease in size and granularity under the influence of thyroxine(1,2). The thyrotrophic pituitary hormone (TSH) exerts, besides stimulating the thyroid gland, a direct action on tissues which has been demonstrated in thyroidectomized animals(3,4,5). Injection of TSH into thyroidectomized guinea pigs is followed by mobilization of fat from normal depots. This tissue fat is replaced by a mucinous substance characterized by a content of acid mucopolysaccharides. The fat mobilizing principle is inhibited by thyroxine(6). Like TSH, somatotrophin (STH) has a direct effect on connective tissue. Various experiments have shown that STH stimulates fibroblast proliferation and formation of collagen (7). In the present work the effect of thyroxine, TSH and STH on number of mast cells and concentrations of hexosamine and hydroxyproline in the skin of dwarf mice has been studied. Hexosamine is an important constituent of acid mucopolysaccharides in the ground substance of connective tissue and was taken as a measure of concentration of these compounds. As collagen is the only protein in connective tissue which contains hydroxyproline, this amino acid was taken as an expression of amount of collagen.

Material and methods. Adult dwarf mice and their normal siblings were used. The dwarf mice are born with hypoplastic pituitary glands(8) which seem devoid of acidophil cells producing STH and basophil cells producing TSH(9). Dwarf mice are the only laboratory animals known to have pituitary (secondary) myxedema (with no or very little TSH and STH production). They were selected for the experiment because of this abnormality. In the first experiment, dwarf mice were divided into 5 groups with 5-16 animals in each group (no normal mice) (Fig. 1). The animals were injected intraperitoneally daily for 10 days. Group I: thyroxine (Roche) 0.1 ml (0.1%), II: TSH (Armour) 0.017 USP unit in 0.1 ml sterile water, III: STH (Armour) 0.5 mg in 0.1 ml sterile water, IV: Phyol‡ (Alfred Benzon) = STH 0.5 mg in 0.1 ml sterile water, V: physiological saline, 0.1 ml. On the 11th day the animals

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* Supported by grants from Danish Rheumatism Assn. and Mr. and Mrs. Reinholdt W. Jorck's Fn.

† Fellow of Helen Hay Whitney Fn. N. Y.

‡ Kindly supplied by Prof. Tage Kemp.

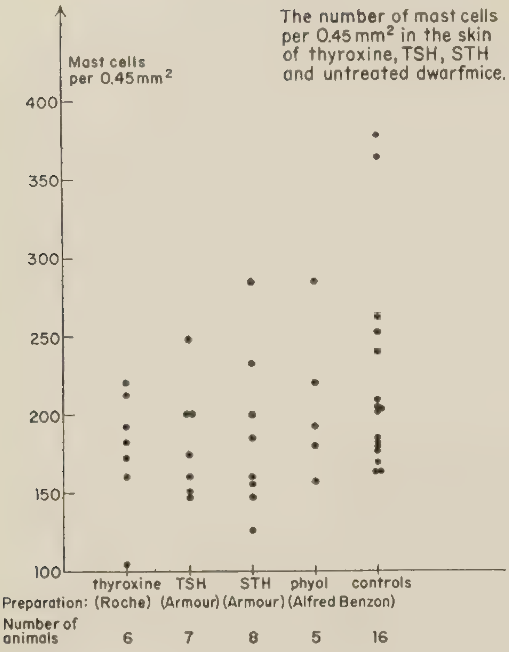

FIG. 1.

were killed and small pieces of skin from the dorsum removed and fixed in a 4% solution of lead subacetate. After dehydration and embedding in paraffin the tissue was cut into slices 10 μ thick (10^{-6}m) and stained for 30 minutes with toluidine blue (0.5% in water). The mast cells of the corium subjacent to the epithelial layer were then counted. An area of at least 0.45 mm² was counted. In the second experiment animals were divided into 3 groups of dwarf mice and 3 groups of their normal siblings with 7-10 animals in each group (Table I). Injections were carried out as in the first experiment except that STH preparations were not given. 100-200 mg of skin-samples were removed as before and dried. Hexosamine analyses were carried out by the method of Elson and Morgan(10), modified as previously described by Kirk and Dyrbye(11) and Dyrbye(12). Hydroxyproline content was determined by Martin and Axelrod's(13) modification of Neuman and Logan's(14) method.

Results. Mast cell countings showed a considerable scatter in all 5 groups (Fig. 1). Statistical examination was attempted. None of the treated groups differed significantly from the control group.

The results are listed in Table I. Statistical calculations showed that the values of hexosamine and hydroxyproline for control dwarf mice and control siblings did not differ significantly from each other.

Hexosamine values for the dwarf mice treated with TSH (Armour) were significantly higher than those of non-treated dwarf mice (t-value + 3.17). This accords with previous findings that TSH increases the quantity of metachromatic substances in the connective tissue. On the other hand, hexosamine values in the group of thyroxine-treated mice were significantly higher than in the group of control mice (t-value + 2.53). Hexosamine values in the other groups showed no significant difference. As regards hydroxyproline, none of the treated groups differed significantly from controls. Comparing dwarf mice treated with TSH with dwarf mice treated with thyroxine, however, we found that in the former group the skin showed a hydroxyproline content statistically higher than that of the latter (t-value + 3.11). These results suggest that thyrotrophic hormone stimulates and thyroxine inhibits formation of collagen fibrils.

Summary. Dwarf mice and their normal

TABLE I. Content of Hydroxyproline and Hexosamine in Skin of Untreated, Thyroxine (Roche) and TSH-Treated (Armour) Dwarf Mice and Their Normal Siblings.

	Hexosamine, mg/g dry tissue			Hydroxyproline, mg/g dry tissue		
Group	Mean value	Stand. dev.	No. of animals	Mean value	Stand. dev.	No. of animals
Dwarfs, thyroxine	3.7	.74	9	13.1	2.5	8
Mice, "	3.9	1.09	9	17.6	4.1	8
Dwarfs, thyrotrophic hormone	4.3	.85	10	18.2	3.9	8
Mice, " "	3.3	.87	8	19.5	6.5	7
Dwarfs, controls	3.2	.71	10	15.4	2.1	10
Mice, "	2.8	.80	10	15.0	3.7	9

siblings were given thyroxine, thyrotrophic and somatotrophic hormones or physiological saline intraperitoneally. In the skin the mast cells were counted and concentration of hexosamine and hydroxyproline were determined. The number of mast cells did not show any significant variation in the different groups. Concentration of hexosamine in the skin of thyrotrophin-treated dwarf mice was significantly higher than concentration in control dwarf mice. Hexosamine values in the group of thyroxine-treated mice were higher than in the group of control mice. Hydroxyproline content was significantly higher in the skin of thyrotrophin-treated dwarf mice than in thyroxine-treated dwarf mice.

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Received July 2, 1959. P.S.E.B.M., 1959, v102.

Influence of Exercise, Androgen Administration and "Atherogenic" Diet on Hematologic Changes in Pullets.* (25270)

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It is well known that the red blood cell count and hematocrit values of humans, mammals and avian species are much higher in males than in females. Juhn and Domm(1) showed that red blood cell differences between the sexes in chickens occurred after puberty and only in males in association with androgen secretion by testes. Gonadectomy of males prevented red blood cell count from differing significantly from that of females. Despite seasonal variations in the number of erythrocytes it was noted that adult male doves and pigeons had a higher count than adult females in all seasons(2). The present study was undertaken to determine the effects of exercise, androgen and an "atherogenic" diet on the erythrocytes, hematocrit and spleen size of pullets.

Methods. Six-week-old New Hampshire White Leghorn pullets were used in an experiment of approximately 11 weeks. The pullets were divided into 5 groups. Controls were fed plain mash. A second group was fed the same diet plus 2% cholesterol and 5% cottonseed oil. The third group, in addition to the diet of Group II, was exercised for one hour daily. Group IV received the same diet as II, was exercised, and injected with 1.25 mg testosterone propionate ("Oreton")‡ intramuscularly daily. We have found this dose of testosterone to be replacement therapy for castrated males. The last group was treated in the same fashion as the 4th, but no exer-

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* Supported by grants from Nat. Heart Inst. and Washington, D. C. Heart Assn., D.C.

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‡ We are grateful to Drs. Edward Henderson and Richard McCormick of Schering Corp., for generous supply of Oreton.

TABLE I. Hematologic Changes in Pullets by Exercise, Androgen and "Atherogenic Diet."

Group	Regimen	No. birds	RBC (mill./mm^3)	Hematocrit (%)	Spleen wt (g)
I	Control (plain mash)	12	2.79 ± .06	31.0 ± .7	3.39 ± .2
II	Mash + Chol + C.O.	9	2.79 ± .1	31.0 ± .8	3.31 ± .2
III	*Idem* + exercise	7	2.83 ± .1	30.0 ± 1.5	3.04 ± .5
IV	*Idem* + T.P.	8	3.15 ± .1*	35.0 ± 2.0†	1.93 ± .2*
V	Mash + Chol + C.O. + T.P.	10	3.25 ± .1*	37.0 ± .8*	2.56 ± .3*

± S.E. of mean. Chol = Cholesterol; C.O. = Cottonseed oil; T.P. = Testosterone propionate.

* Significant .01. † Significant .02.

cise. The birds were exercised on a treadmill designed for this purpose consisting of a cylindrical structure enclosed in wire mesh. Diameter of the treadmill was 23½ inches, length 33 inches and outside perimeter 5 feet 10 inches. Speed was regulated at 6 ± 1 rpm. The animals were exercised in this apparatus for one hour daily, or approximately 700 yards. Red blood cell and hematocrit determinations were carried out using heparinized blood obtained from wing veins. Erythrocyte counts were made using a Spencer Bright-Line Haemocytometer. Blood was diluted with Wiseman's diluting fluid which contained 50 mg of phloxine, 5 cc of neutral formalin and 75 cc of Ringer's solution(3). Hematocrit values were determined by centrifugation of blood for 30 minutes in Wintrobe hematocrit tubes. At conclusion of the experiment all animals were sacrificed and spleen weights taken.

Results. Those birds treated with testosterone propionate were the only ones significantly different from the controls regarding hematological findings. There was an increase in erythrocyte counts and hematocrits, and a decrease in spleen weights. Addition of cholesterol to the diet, or submitting the birds to exercise, or a combination of these, did not appear to effect the above 3 indices (Table I).

Discussion. These data indicate that the hematologic changes produced probably resulted from the action of testosterone propionate, as the other variables, cholesterol and cottonseed oil as well as exercise, produced no significant changes in red blood cell count, hematocrit or spleen weight. Increase in hematocrit in Groups IV and V is consistent with increased red blood cell count and may represent some degree of hemoconcentration. Normal red blood cell counts in adult male chickens average 3.25 million/mm^3(4). In the present experiment it should be noted that pullets receiving testosterone propionate had values similar to adult male birds.

Decrease in spleen weight of the birds in Groups IV and V and the increased number of red blood cells in circulation suggest that the additional erythrocytes have come from spleens. It has been shown that the spleen of fowls is utilized as a storage place for erythrocytes(5).

Since there is evidence that cholesterol is a precursor of steroid hormones of the adrenal gland(5), it may be postulated that an increase in cholesterol in diet might lead to increased production of adrenal androgens. If this should occur, in view of the results obtained in this experiment, one might expect hematological changes also. No such changes were noted in this study. The red blood cell count in those birds receiving cholesterol was the same as in the controls on plain mash.

It appears therefore, that amount of cholesterol in the diet does not influence the hematological picture in pullets. There is reason to believe that excess cholesterol is either deposited on vascular walls and/or is metabolized to cholic acid and excreted in bile. This can be supported by the abundance of bile found at autopsy in all birds fed a diet rich in cholesterol.

Summary. Pullets receiving testosterone propionate demonstrated a significant increase in red blood cell count and hematocrit and a decrease in spleen weight.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Pancreatic Collagenase, A New Enzyme.* (25271)

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Ziffren and Hosie(1) claimed that pancreatic juice possessed ability to digest collagen fibers. Straumford and Hummel(2) showed that this activity was inhibited by soybean trypsin inhibitor. More recently, Oken and Boucek(3) claimed that impure pancreatic elastase solubilized collagen fibers, whereas the crystalline enzyme did not. Investigations into collagenolytic enzymes have always been questionable because of the difficulty of assaying an enzyme using an insoluble substrate. Recently Gallop(4) has shown that only bacterial collagenase will reduce the viscosity of procollagen derived from swim-bladder tunica of the carp. We studied the effect of pancreatic extracts and enzymes upon viscosity of acid soluble collagen of calf skin.

Material and methods. Purified acid soluble collagen was prepared from 0.5 M acetic acid extracts according to Gross and Kirk(5). This material was lyophilized, and solutions of 0.2 M acetate buffer (pH 5.5) were made to contain 15 mg/ml of procollagen. These solutions were homogeneous with respect to electrophoresis and in amino acid and hexosamine content were as reported by Gross and Kirk (5). Crystalline trypsin, elastase, chymotrypsin and relatively purified hyaluronidase were obtained from Worthington. Human pancreatic juice was obtained from a patient with freely flowing fistula. Aqueous extracts of "Bridase," a whole, desiccated, defatted, activated preparation of swine pancreas provided by Mr. Levin of VioBin, were prepared by extracting 25 to 100 mg of "Bridase" with 5 ml of 0.2 M acetate buffer (pH 5.5) in a glass homogenizer at 4°C. After centrifugation, about 45% of total weight of tissue had been solubilized into the clear supernatant. Concentration of this material was expressed in terms of mg of "Bridase" initially mixed with 1 ml of solvent. Assay of enzyme activity was as follows: 4 ml of substrate solution (15 mg/ml of acetate buffer) was warmed to 20°C, added to an Ubbelohde viscosimeter in which water had a flow time of about 70 seconds. To this viscosimeter was added 1 ml of buffer containing 1 mg of various enzymes described above, or 1 ml of "Bridase" extract. After manual mixing for a few minutes, the solution was incubated for 75 minutes and flow time determined. The difference between relative viscosity of this solution and that of the control previously found, using boiled enzyme solutions, was expressed as percent of initial viscosity reduced by 75 minutes incubation with the enzyme.

Results. The effect of 1 mg each of crystalline trypsin, chymotrypsin and elastase, as well as the effect of 1 mg of hyaluronidase and 1 ml of human pancreatic juice upon relative viscosity of acid soluble collagen was determined. Similarly, the effect of the aqueous extract of 15 mg/ml of "Bridase," with and without addition of 1 mg each of either soybean, ovomucoid and lima bean trypsin inhibitor was also measured, and the results expressed in terms of per cent viscosity reduced is shown in Table I. The pancreatic extract was uniquely able to reduce 50% of the viscosity of acid soluble collagen. This activity was destroyed by boiling and was independent of addition of 3 kinds of trypsin inhibitor. Amount of inhibitor added was in excess as shown by the method of Rhodes *et al.*(6).

Fig. 1 shows that after 75 minutes incuba-

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* Supported by grants from N.I.A.M.D. and VioBin Corp.

TABLE I. Effect of Various Enzyme Preparations upon Relative Viscosity of Acid Soluble Collagen.

Incubation mixture (75 min.)	Relative viscosity Exp.	Control	Viscosity reduced, %
Substrate +			
Buffer	14.57	14.57	0
Trypsin (1 mg)	"	14.58	0
Chymotrypsin (1 mg)	"	14.57	0
Elastase (1 mg)	14.53	14.50	0
Hyaluronidase (1 mg)	14.58	14.58	0
Pancreatic juice (1 ml)	"	"	0
"Bridase" (15 mg)	7.39	14.50	50
" + soybean inhib. (1 mg)	7.35	14.60	"
" + lima bean inhib. (1 mg)	7.30	14.61	"
" + ovomucoid inhib. (1 mg)	7.33	14.56	49

tion with both a 15 mg/ml and a 20 mg/ml solution of "Bridase," most of the viscosity loss produced by this preparation was complete. With lesser amounts of "Bridase," longer periods of time were required for complete reaction. The data presented in Fig. 1 were obtained in the presence of soybean trypsin inhibitor. Previously, pancreatic washings have been shown to be unable to reduce the viscosity of acid soluble collagen solutions. It may also be seen from this figure that the % viscosity reduced is directly proportional to concentration of the "Bridase" used.

The effect of various concentrations of "Bridase" after 75 minutes incubation at 20°C with acid soluble collagen upon % viscosity reduced is shown in Table II. Prolonged exposure of acid soluble collagen solutions to 37°C results in their polymerization to form collagen fibers(5,7). After incubation of the collagen solution with the enzyme as described above, these solutions were then heated to 37°C for 5 minutes. The absorbance of resulting fibers was determined in a "Spectronic-20" spectrophotometer. These results, expressed in terms of percentage difference between absorbance of control and enzymatically pretreated substrate solutions, are given in Table II. The loss of viscosity described above was paralleled by a decrease in the fiber-forming property of the substrate solutions. Soybean trypsin inhibitor was without effect upon the fiber forming inhibition of "Bridase" extracts.

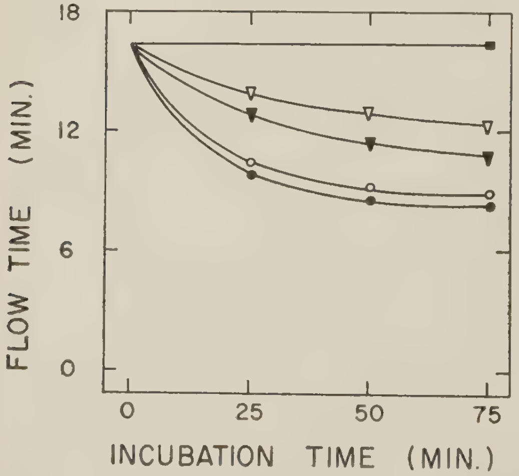

FIG. 1. Flow time (min.) of acid soluble collagen solutions after various periods of incubation with 20 (●), 15 (○), 10 (▲) and 5 (△) mg of "Bridase." Effect of a previously boiled preparation of 15 mg/ml of "Bridase" is shown by the top (■) line.

TABLE II. Viscosity Reduction and Fiber Formation Inhibition of Acid Soluble Collagen by Treatment with "Collagenase."

Incubation mixture (75 min.)	Viscosity reduction %	Fiber reduction
Substrate alone (12 mg/ml)	0	0
+ 5 mg Bridase	26	33
+ 10 " "	39	38
+ 15 " "	49	49
+ 20 " "	52	51
+ *Idem* + soybean inhib.	51	50

The effects of "Bridase" upon viscosity of serum albumin, casein, egg albumin and Knox I.V. gelatin in the presence of excess trypsin inhibitor were also studied. No reduction in viscosity of these proteins was observed.

Conclusions and summary. From these

data, it is apparent that whole activated pancreas, unlike pancreatic juice, contains a thermo-labile material which can reduce by 50% both viscosity and ability to form fibers of acid soluble collagen solutions. This activity is not affected by various trypsin inhibitors, nor is it duplicated by trypsin, elastase, chymotrypsin or hyaluronidase alone. This activity may be due to a new enzyme in the pancreas, a collagenase. In view of lack of activity of this enzyme upon serum and egg albumin, casein and gelatin, this enzyme would seem to be reasonably specific for acid soluble collagen.

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Received July 10, 1959. P.S.E.B.M., 1959, v102.

Mobilization of Neutrophils into Peritoneal Fluid Following Intraperitoneal Injection of Bacterial Endotoxins.* (25272)

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Contrary to frequent reports that a 0.9% NaCl solution provokes local neutrophilia when injected intraperitoneally, we found that this does not occur in rats if the saline solution is sterile and non-pyrogenic. Moreover, a solution to which a small amount of a bacterial polysaccharide complex derived from Pseudomonas (Piromen) had been added, produced a marked neutrophilia when injected intraperitoneally(1). Therefore, it was of interest to determine whether or not cellular reactions of peritoneal fluid can be used as a sensitive indicator to detect presence of small amounts of certain bacterial substances.

Methods. Male rats of Sprague-Dawley strain (Holtzman Co.) and weighing 180-220 g were used. All rats received an intraperitoneal injection of 10 ml sterile, non-pyrogenic saline (Baxter Lab.). Solutions were injected at room temperature and the rats allowed food and water *ad lib.* during experiment. Animals were challenged by graded amounts of bacterial extracts added to the saline solutions immediately before injection. Three bacterial extracts were used: 1) Piromen,[†] a Pseudomonas polysaccharide complex (Baxter Lab.), 2) Staphylococcus A lipopolysaccharide (Difco Lab.), and 3) *E. coli* 026: B6 lipopolysaccharide (Difco Lab.). Control animals received only sterile saline solution, free of pyrogens. After 5 hours rats were sacrificed, then 10 ml heparinized 0.9% saline solution was injected intraperitoneally. This was done because most of the original 10 ml of solution is absorbed from the cavity by 5 hrs. Animal's abdomens were massaged gently for one minute. Then an incision was made into the peritoneal cavity and the fluid containing a suspension of cells was collected with medicine dropper. The fluid was centrifuged to concentrate the cells which were then resuspended in homologous serum. Smears were made using a brush technic(2), and stained by modified Wright-Giemsa stain(3). In the unstimulated state the peritoneal fluid contains mononuclear cells (macrophages and lymphocytes), eosinophils and mast cells, but is virtually devoid of neutrophils. Percentages of various cells, excluding mast cells, were computed. On the basis of previous experience, it was decided that appearance of 5% or more neutrophils in the peritoneal fluid

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* Supported by grant CY-3071 from USPHS.

[†] We are indebted to Dr. L. G. Ginger, Baxter Labs., for Piromen used.

TABLE I. Appearance of Neutrophils in Peritoneal Fluid 5 Hours after Injection of Bacterial Extracts.

Treatment	Dose, µg/rat	Positive response/total No. of rats
Piromen	Saline control	0/16
	1×10^{-4}	0/9
	$5 \times$ ”	0/4
	1×10^{-3}	6/12
	Graded doses from 3.16×10^{-3} to 3.16×10	91/92
Staphylococcus A polysaccharide	Saline control	0/8
	1×10^{-5}	0/4
	1×10^{-4}	4/8
	$5 \times$ ”	5/7
	Graded doses from 1×10^{-3} to 1×10^{4}	31/31
E. coli 026:B6 polysaccharide	Saline control	0/4
	5×10^{-4}	0/4
	Graded doses from 1×10^{-3} to 1×10^{3}	28/28

should constitute a positive response.

Results. Table I summarizes effects following injection of graded doses of the 3 bacterial derivatives used. Both Piromen and *E. coli* extracts evoked a measurable response following injection of .001 µg of material, and Staphylococcus A extract was potent when as little as .0001 µg was injected. Control animals which received injections of 10 ml sterile, non-pyrogenic saline were all negative in regard to neutrophil mobilization. Once a dose necessary to elicit a maximum response was reached, practically all animals responded irrespective of dose. The neutrophils which appeared in the fluid were mature and resemble those seen in circulating blood.

Discussion. The massing of neutrophils in the peritoneal cavity following injection of as little as .0001 µg of a bacterial lipopolysaccharide offers proof of the extreme sensitivity of the rat to certain bacterial derivatives. This is even more remarkable since the rat has been thought to be singularly unresponsive to such substances(4). The official method for detection of certain bacterial contaminants in solutions is based on the febrile response in rabbits(5). Unfortunately, the rabbit has a labile thermo-regulatory mechanism and frequently gives false positive results(6). The alteration of peripheral white blood cell count also has been suggested as a means of detecting pyrogens in fluids(7). Some investigators have called attention to the peripheral leukopenia, noted shortly after injection of pyrogen(8) while other authors noted, in addition, that pyrogenic substances evoke a neutrophilic leukocytosis several hours after their administration(9). The need for serial white blood cell counts coupled with the normally fluctuating nature of the leukocytic picture constitute difficulties in assessing such changes. In contrast, examination of peritoneal fluid offers some distinct advantages over previous methods. Since the unstimulated animal has practically no neutrophils in the peritoneal cavity, their presence there, even in small numbers, is quickly detected and easily quantitated. Secondly, one can inject relatively larger volumes of fluid intraperitoneally than intravenously or subcutaneously. Also, only a single determination/animal is necessary. Preliminary studies showed that the neutrophil response can be tested in other laboratory animals such as the mouse. It will be necessary to test the response to many types of substances to ascertain specificity of the peritoneal fluid response to bacterial products. Such work is now in progress. Glucose, for example, in contrast to published observations, was ineffective in mobilizing neutrophils in the peritoneal fluid(10), provided it was administered in pyrogen free solutions.

Although our report is concerned with percentage changes in neutrophils only, quantitative studies involving absolute cell counts have been done and will be reported. These studies reveal that very high doses of bacterial polysaccharides may diminish total numbers of neutrophils which can be mobilized, although the response is not abolished entirely.

Since the neutrophil plays a primary role in defense of the organism against certain bacteria, it is perhaps not surprising that one can stimulate the mobilization of these cells by such minute amounts of bacterial material. Moreover, the rationale for using mobilization of neutrophils to detect and assess bacterial products gains additional support from the work of investigators showed the close relationship between neutrophils and pyrogens (11,12). It will be of interest to determine whether peritoneal fluid response can be equated with febrile response.

Summary. Injection of certain bacterial polysaccharides into the peritoneal cavity of rats results in a local neutrophilia which can easily be determined and quantitated. Preliminary findings suggest the possibility of using this response to detect the presence of certain bacterial derivatives in solutions.

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Received July 20, 1959. P.S.E.B.M., 1959, v102.

Thyroiditis Resulting from Administration of Excess Iodine to Hamsters with Hyperplastic Goiters.* (25273)

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When physiological amounts of iodine are administered to hamsters which have hyperplastic goiters produced by an iodine-deficient diet(1), colloid rapidly accumulates in the follicles to bring about the morphological picture of colloid goiter. The suitability of the hamster for studies on the thyroid prompted us to investigate the effects of large amounts of iodine in order to reproduce, if possible, the state of *Jodbasedow.* It will be recalled that T. Kocher(2) introduced this term in 1910 to refer to the syndrome of clinical hyperthyroidism occurring in persons having endemic goiters to whom iodine had been administered. The only clear-cut example of this phenomenon in the experimental animal was reported by Webster and Chesney(3) 30 years ago when they administered large amounts of iodine to rabbits with hyperplastic goiters produced by a cabbage diet. Such animals lost weight, developed increased metabolic rates, and died; at autopsy their thyroid glands exhibited a patchy reaccumulation of colloid, but not true colloid goiter. When hamsters with hyperplastic iodine-deficient goiters were given large amounts of iodine, as will be reported herein, an inflammatory reaction developed in the gland, giving rise in a short time to the picture of sub-acute thyroiditis. It is the purpose of this communication to describe the evolution of this process in hyperplastic glands produced by iodine-deficiency or goitrogens(4).

Materials and methods. A total of 28 hamsters weighing approximately 100 g were observed on 3 different diets: 1) an iodine-deficient ration composed of whole ground corn, 76; wheat gluten, 20; calcium carbonate, 2; and sodium chloride, 2; and 500 mg of a complete vitamin mixture per kilo. 2) thiouracil and 3) potassium thiocyanate, added to batches of the basal diet at levels of .3% and .5%, respectively. Sixteen animals were maintained on the iodine-deficient diet for periods up to 30 weeks. The thiouracil-containing diet was administered to 6 animals for 3-6 weeks, while potassium thiocyanate was

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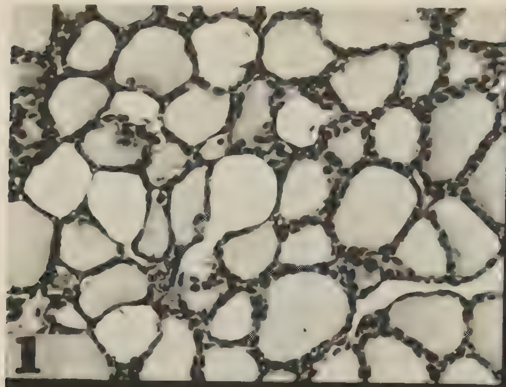
* Supported in part by grant-in-aid from USPHS.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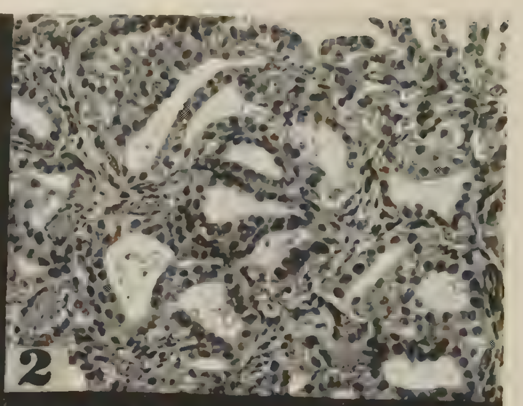
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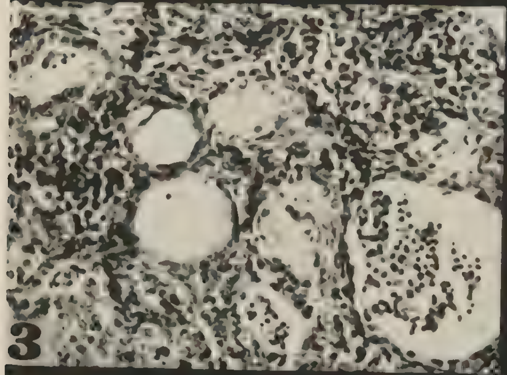
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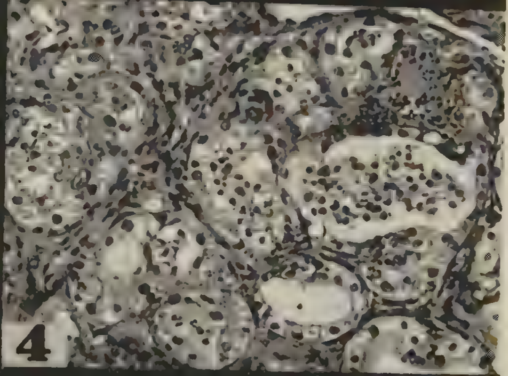
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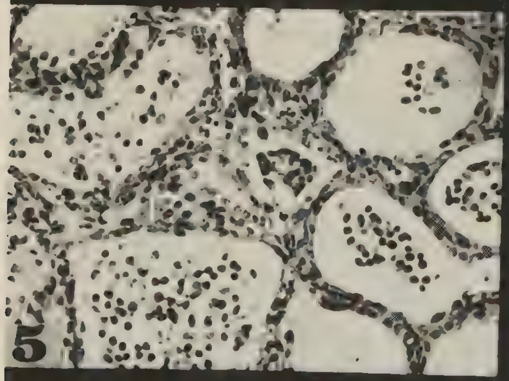
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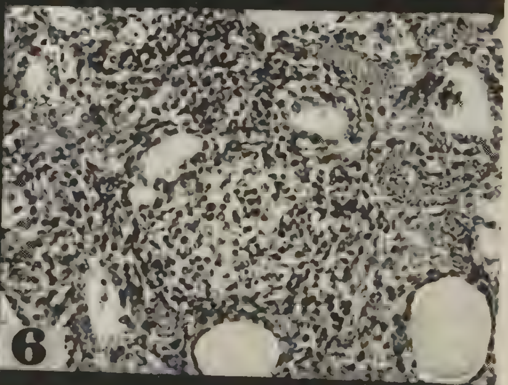
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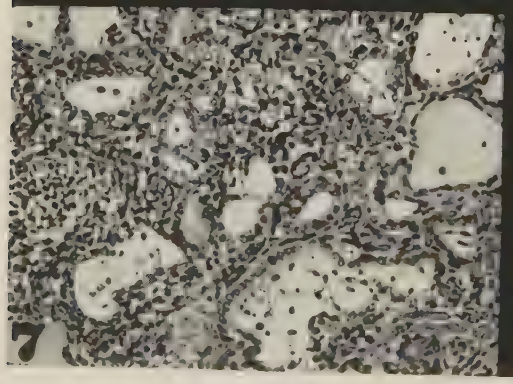
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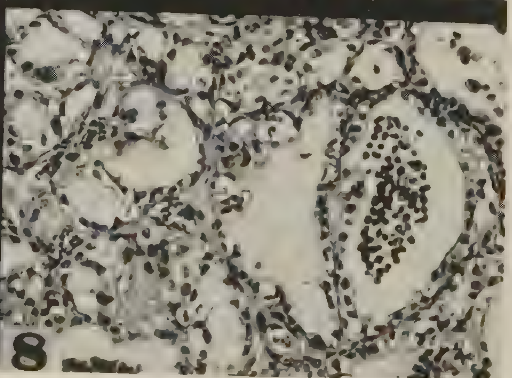
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fed to 6 hamsters for a period of 5 weeks. When hyperplasia was well-established, excess iodine was administered as potassium iodide, 10 mg intraperitoneally per day, for periods up to 2 weeks. Six normal animals receiving this amount of iodide were also studied. Animals were killed serially during the period of iodine administration. Tissues were prepared for histological examination in routine manner.

Observations. Before describing alterations produced by excess iodine it will be advantageous to review the microscopic appearance of normal and hyperplastic glands, together with changes following administration of physiological amounts of iodine(1) or cessation of administration of goitrogens(4). The normal thyroid of the hamster is composed of colloid-filled follicles, averaging 45 μ in diameter and lined by flattened or low cuboidal cells. Adipose tissue is found within the gland; blood vessels are not prominent. No cellular infiltration is seen save an occasional mast cell. After hyperplasia, produced by either iodine deficiency(1) or a goitrogen(4), has developed, colloid disappears; blood vessels are more prominent and epithelial cells increase in size and number. In our experience no interstitial infiltration is seen under such circumstances. Following the introduction of physiological amounts of iodine or removal of the goitrogen from the diet colloid begins to reaccumulate in the follicle. The epithelial cells become more flattened; the follicle increases in size. Vascularity is decreased. No interstitial inflammatory reaction is found; an occasional mononuclear cell filled with fat may be present in the lumen of some follicles.

After administration of large amounts of iodine to hamsters which have been on an iodine-deficient diet, the earliest change appears after 24 hours. This consists of the presence of polymorphonuclear leukocytes in the lumens of the follicles. At the beginning, only an occasional structure is involved; however, after several days many contain cellular exudate. Such follicles are usually devoid of colloid. Cells which doubtless represent desquamated thyroid epithelial structures are found among the leukocytes. At this early stage, while the blood vessels are still prominent as a result of the preceding hyperplasia, leukocytes are found adherent to capillary walls as though they were making their way through these structures. Relatively little interstitial infiltration is present at this time, however. Those follicles which are free of leukocytes contain a pale-staining eosinophilic material and have increased somewhat in diameter. It is difficult to find necrotic epithelial cells, though an occasional cell with a pyknotic nucleus is made out. At the end of a week the cellular infiltration within the follicles is more extensive. Such collections are made up of polymorphonuclears and round cells. A definite interstitial infiltration is found between the follicles at this time; the cells composing this exudate are primarily mononuclear, *i.e.*, lymphocytes together with an occasional plasma cell. By now the blood vessels are less prominent in a fashion similar to the decrease in vascularity seen after physiological amounts of iodine. The follicles have increased in size, yet not nearly to the diameter which would have been expected as a result of the administration of physiological amounts of the element. Some are even devoid of colloid at this time. At the end of 2 weeks the acute inflammatory reaction within the follicles has disappeared; a more chronic though less intense form in which mononuclear cells predominate is found. A moderate inter-

All sections stained with H. and E. All ×200.

FIG. 1. Normal; clear areas are fat.

FIG. 2. Hyperplasia after 28 wk on iodine-deficient diet; note increase in number and size of cells and granular material in lumens of follicles. This is control for animals shown in Fig. 3-7.

FIG. 3. Two days after excess iodine administration to animal with hyperplasia shown in Fig. 2; note leukocytes within follicles, absence of colloid and interstitial infiltration.

FIG. 4. Four days after excess iodine administration.

FIG. 5. Six days after excess iodine administration.

FIG. 6 and 7. Ten days after excess iodine administration.

FIG. 8. Six days after administration of excess iodine to animal which had hyperplasia resulting from potassium thiocyanate administration. Follicles have not accumulated excess colloid; note cells within follicle.

stial inflammatory reaction remains. The change has been followed for 2 weeks at which time the inflammatory reaction is still present.

Hyperplasia can be produced in the hamster by a goitrogen such as thiouracil(4); moreover, the follicles fill up with colloid when the drug is removed from the diet. The effect of large amounts of iodine on hyperplastic glands produced when thiouracil and potassium thiocyanate are fed with the iodine-deficient diet, was therefore studied. When 10 mg doses of potassium iodide are given to animals receiving thiouracil, the cellular infiltration of the follicles and interstitial tissues appeared to be identical with that described in iodine-deficient hamsters. The follicles, moreover, do not contain as much colloid as those from animals which have received physiological amounts of iodine. Since colloid cysts are seen more commonly in thiouracil-treated animals than in those on an iodine-deficient ration before realimentation with iodine, the lack of involution was not as uniform in this group as in the preceding one.

When excessive amounts of iodide are administered to hamsters with hyperplastic glands produced by potassium thiocyanate administration, polymorphonuclear leukocytes soon appear within the lumens of the follicles and in the course of a week cellular infiltration is found in the interstitial tissues as well. The increase in size of the follicles as a result of colloid accumulation is not as great as that seen when physiological amounts of iodine are administered.

Discussion. The original intent of these experiments was to produce a syndrome analogous to *Jodbasedow* by administration of large amounts of iodine to hamsters with hyperplastic thyroid glands which had developed as a result of iodine deficiency. We hoped to repeat Webster and Chesney's observation(3) on the response of rabbits with cabbage goiter to administration of excess iodine. Although no metabolic studies were performed on the animals herein reported, the hamsters did not lose weight or die in the manner of Webster and Chesney's rabbits. The pathogenesis of alteration which has been described in the hamster thyroids, that is, a form of thyroiditis, cannot be explained, though the lesions deserve some comment.

First, these experiments show that the effects produced by physiological amounts of iodine on the hyperplastic gland are different from those resulting from massive doses. We have now studied the thyroid glands of over a 100 hamsters which have developed colloid goiters resulting from the involution of hyperplasia produced by either iodine deficiency(1) or administration of several different goitrogens(4). In each case the follicles become distended with large amounts of colloid. More important, no inflammatory reaction within the follicles has been seen, nor is there evidence of interstitial cellular infiltration. In contrast, when large amounts of iodine are administered, as in these experiments, not nearly as much colloid accumulates in the follicles; the inflammatory reaction which develops has been described above. To our knowledge a lesion such as this has not heretofore been produced in the thyroid gland in an experimental animal by administration of large amounts of iodine. The development of painful swelling of the thyroid gland, called by some "iodine thyroiditis," has been mentioned from time to time in the human and is said to result from excessive iodine administration(5). Histological studies of such glands have not been reported. However, one sometimes sees an intrafollicular cellular infiltration in the thyroid glands of patients with Graves disease to whom iodine has been administered pre-operatively. When large amounts of iodine are given to normal animals, no prominent change in basal metabolic rate has been detected(6), though a decrease in uptake of I^{131} has been reported (7). In our own experience no morphological alterations occur after administration of large amounts of iodine to normal hamsters. Some observations may be cited to indicate that iodine has certain known, as well as doubtless unknown, actions on the activity of the thyroid epithelial cells, particularly when administered in excessive amounts(8). For instance, iodide appears to interfere with the action of TSH on the thyroid gland of hypophysectomized rats, since the hyperplasia produced by thyrotropic hormone in such animals is decreased by large amounts of potassium iodide. Moreover, iodine decreases the inactivation of TSH by thyroid tissue *in vitro*.

An inflammatory reaction in the thyroid glands of experimental animals has been produced by several technics; the most striking alterations follow radiation or auto-immunization procedures. Treatment of rats with appropriate amounts of I^{131}(9) leads to damage of thyroid epithelial cells and an inflammatory reaction not unlike the changes described above. So, too, the descriptions of auto-immune thyroiditis in several species(10) are certainly reminescent of the alterations we have observed here, so much so that we are currently attempting to produce thyroiditis in hamsters by injection of homologous thyroid tissue with an adjuvant and are examining the sera of animals with iodine thyroiditis for antibody to thyroglobulin. We are unable to explain the pathogenesis of the changes described herein. Certain possibilities such as the deleterious effects of accumulation of large amounts of iodine in a depleted gland, or production of excessive amounts of thyroid-active materials within the gland come to mind. Further study of this problem may help to understand the phenomena of *Jodbasedow*(2) and "iodine thyroiditis"(5) in man.

Summary. Administration of large amounts of iodine to hamsters which have hyperplastic goiters produced by an iodine-deficient diet or by a goitrogen results in a morphologic response which is different from that encountered when iodine is given in physiological amounts. An acute inflammatory reaction and a diminution of the accumulation of colloid are found, neither of which has been observed in animals treated with smaller amounts of iodine.

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Received July 28, 1959. P.S.E.B.M., 1959, v102.

Synthesis, Cytopathogenicity, and Modification of Avian Encephalomyelitis Virus (AEV) in Chick Kidney Cell Culture.* (25274)

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Multiplication of AEV in minced whole chicken embryo tissue culture has been reported(1). Owing to the low titer obtained the method is of limited value. This communication reports successful propagation of the virus in monolayer chick kidney cell culture, cytopathogenic changes produced in the cell culture, and modification of virulence of the virus for chicks.

Materials and methods. Virus. The seed virus was the brain homogenate from 6th chicken embryo passage. It had previously undergone 16 passages in chicks, after being received as the 135th chick-brain passage virus.† The titer of the virus was $10^{-5}/0.05$ ml when inoculated intracerebrally into chicks. *Cell culture.* Monolayer chick kidney cell culture was prepared from 1-week-old chicks (2). *Passage of virus.* The growth medium (2) was replaced by 0.1 ml virus and 0.9 ml growth medium containing 0.07% sodium bicarbonate. Serial passage was made every 3

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* Aided in part by grants from Nat. Inst. Health and Am. Poultry and Hatchery Federation.

† Kindly supplied by Dr. Henry Van Roekel, Dept. of Vet. Sci., Mass. Agric. Exp. Station, Amherst.

days up to 5th passage and every 2 days thereafter up to 10th passage. *Titration.* The 10th passage virus was titrated in 1-week-old chicks by intracerebral (IC) inoculation(3) and in a cell culture system using 4 chicks and 4 culture tubes for each virus dilution. *Identification of passage virus.* Four 1-week-old chicks were inoculated IC with 0.05 ml of the 10th passage virus. Brain, proventriculus, and ventriculus were taken for histopathologic study after definite signs of infection were recognized. Virus neutralization tests in a cell culture system were performed with 5 hyperimmune sera prepared by repeated intramuscular inoculation(4) of adult chickens with increasing dosages of chick brain virus and enhanced with embryo brain virus. The preinoculation sera were found by the *in vitro* virus neutralization test to be free of specific neutralizing antibodies. Five normal sera were included as controls. An equal volume of serum and virus dilution mixture was incubated at room temperature for 1 hour, and 0.2 ml of the mixture plus 0.8 ml of serum-free maintenance medium(2) were inoculated into each cell culture tube. Readings were made at 7th day of incubation.

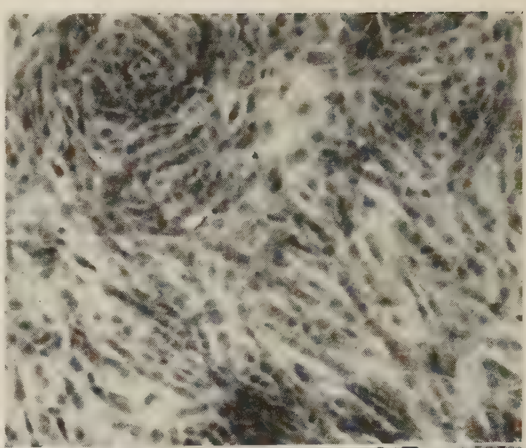

FIG. 1 (top). Chick kidney cell culture. Control. H & E, × 100.

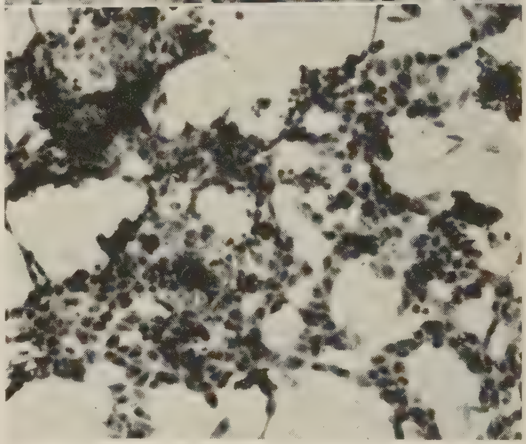

FIG. 2 (bottom). Chick kidney cell culture, 48 hr post inoculation with AEV. H & E, × 100.

Results. Virus synthesis and modification of virus virulence for chicks. The 10th cell culture passage virus titered $10^{-1}/0.05$ ml in chicks and 10^{-8} in the cell culture system. Since the original seed virus titer in chicks was $10^{-5}/0.05$ ml, there would be no virus carried over to the 6th passage. The virus titer in the cell culture can be considered high. Virus synthesis in the system is apparent, but the virus virulence for chicks was attenuated. *Cytopathogenicity.* There were no recognizable cytopathologic changes in the culture up to the 3rd passage. Starting from the 4th passage, definite changes became apparent. They consisted of contraction and rounding of infected cells, cytoplasmic granulation, and cytolysis (Fig. 1 and 2). The changes increased in intensity and incubation period shortened as successive passages were carried on. From the 6th passage the virus was harvested on the 2nd day of incubation. *Identification of passage virus.* All 4 inoculated chicks showed severe torticollis and histopathologic study revealed neuronal degeneration, gliosis and perivascular infiltrates in the brain, and lymphocytic aggregates in the proventriculus and ventriculus musculature which are pathognomonic for AEV infection(3,5). The hyperimmune sera neutralized 10^7 to 10^8 $TCID_{50}$, while normal sera neutralized only 10 to 50 doses. The virus was identified as AEV.

Discussion. AEV preparations for use in research and vaccine production have hitherto been supplied in the form of brain homogenate from infected chicks(3,6) or chicken embryos(7). These preparations contain a large quantity of foreign substances and may be difficult to prepare because the supply of susceptible chicken embryos is often curtailed by widespread occurrence of the infection among breeding stock(8). The cell culture virus may furnish a relatively pure stock for both research and vaccine production. Attenuation of virulence of the virus for chicks by tissue culture may prove to be desirable for immunization purposes.

The diagnosis of an acute AE infection in chicks is thus far mainly based on clinical observations and histopathologic findings. Difficulty in differentiating AE from Newcastle disease and neurolymphomatosis on a histopathologic basis has been encountered. The diagnosis of inapparent infection in breeding flocks by embryo susceptibility test has been employed(8). The test requires 3 weeks to complete and is costly. Collecting representative eggs from large commercial farms and the shipment of them to laboratories may present a problem at times. A one-tube *in vitro* virus neutralization test using 100 $TCID_{50}$ might serve as a simple method for diagnosis of acute AEV infection in chicks and inapparent infection in adults. IC challenge and virus neutralization tests in chicken embryos have been used for evaluation of immunity in inoculated flocks(6,9). The former method is considered too severe and only a fraction of birds can be challenged; the latter method has the disadvantage of requiring a source of susceptible embryos. The virus neutralization test in a cell culture system may serve as a means of evaluating the immunity status of vaccinated flocks without the disadvantages mentioned.

Summary. Virus synthesis, cytopathogenicity, and modification of virulence for chicks, of AEV in chick kidney cell culture after 10 serial passages, have been described. The cell culture virus has been identified by histopathologic changes in inoculated chicks and virus neutralization tests in a cell culture system. The possible application of the findings are discussed.

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Received July 28, 1959. P.S.E.B.M., 1959, v102.

Plaque Assay of *Toxoplasma* on Monolayers of Chick Embryo Fibroblasts.*† (25275)

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The importance of *Toxoplasma gondii* as a parasite pathogenic for man and for many animal species is documented in voluminous literature dealing with various aspects of epidemiology, pathology, clinical findings, diagnosis and treatment(1,2). Relatively less is known about the organism as an independent entity. Its classification as a protozoon is based largely on morphological considerations (3). Although experimental infections of various animals and tissue cultures(2) have been studied in considerable detail, we know nothing about the attributes of the parasite responsible for its extremely fastidious requirements for survival and growth. To date, it has not been possible to propagate *Toxo-*

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* Supported by research grants from NIAID, N.I.H., U.S.P.H.S.

† These studies formed part of a dissertation submitted by senior author to Faculty of Graduate School of Saint Louis University in partial fulfillment of requirements for degree of Ph.D. in Microbiology.

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plasma outside of living susceptible cells, and there is no way of assuring quantitative survival in cell-free media. Therefore, it has been difficult to study its metabolism and mode of multiplication, or, indeed, to establish criteria for viability of the organism. Because, operationally, *Toxoplasma* is an obligatory intracellular parasite, experimental work has largely been patterned after that concerned with viruses. The key to rapid progress in virology has been the gradual evolution of increasingly sensitive and quantitative assay methods. Of special usefulness is the plaque assay described first for bacteriophages(4) and then adapted for use with animal viruses by Dulbecco and Vogt(5). Availability of a similarly quantitative assay for work on *Toxoplasma* would seem to be a prerequisite for systematic investigation of the organism at a fundamental level. The demonstration of cytopathogenic effects produced by *Toxoplasma* in several different kinds of cultured mammalian or avian cells(2) suggested that a plaque system could be developed. Our experiments confirmed this expectation and led to the definition of several quantitative parameters.

Materials and methods. RH strain of Toxoplasma gondii was obtained from Dr. Leon Jacobs of National Institutes of Health. For maintenance, it was subjected to serial intraperitoneal passages in Swiss mice at regular intervals of 4 days. To obtain the organism from infected mice, peritoneal exudate was obtained by injecting 2.5 ml of growth medium (see below) into the peritoneal cavity and aspirating the fluid contents with a syringe. After defibrinating by rolling with glass beads for 5 minutes, the suspension was expelled repeatedly with force through a 27 gauge needle against the inner wall of a test tube to free intracellular parasites. The success of this procedure was verified by microscopic examination which revealed very few intact cells, but millions of extracellular parasites. The same method was used for artificial liberation of intracellular organisms from infected chick embryo fibroblasts. *Chick embryo fibroblast monolayers* (*CEF*) were prepared according to the method of Dulbecco and Vogt(5). Trypsinized cell suspensions were prepared from 9-day-old chick embryos. Growth medium consisted of Hanks balanced salt solution (Hanks BSS) containing 20% horse serum (Ho) and 5% chick embryo extract (EE). For establishment of monolayers, 5 ml of growth medium containing 3 x 10^7 cells were placed in a 50 mm Petri dish. After 15 to 24 hours of incubation in a humidified atmosphere of 3% CO_2 in air at 37°C, confluent sheets were ready for infection. *Infection of monolayers.* After removal of growth medium, the monolayers were infected with 0.5 ml of *Toxoplasma* suspension. Different suspending media were used, and varying periods of time were allowed for adsorption of the organisms, as will be described. Adsorption took place at 37°C. At the end of adsorption period, the monolayers were overlaid with 5 ml of agar medium either directly or after removal of the inoculum and washing with phosphate-buffered saline (PBS). The overlay medium consisted of 0.5% bovine albumin, 0.1% yeast extract, 10% Ho, 5% EE in Earle's balanced salt solution (Earle's BSS), 0.9% agar and neutral red at final concentration of 1:30,000.

Results. Plaque production by Toxoplasma. Discrete round plaques, averaging 0.34 mm in diameter, were first visible on fourth day after infection with *Toxoplasma* suspension. Over the following 4 days, they grew to 1.2 mm in diameter. Centrifugal growth became progressively slower, suggesting more rapid destruction of cells infected initially than later. Because of their small size, up to 500 plaques could be counted easily on a single plate 5 days after infection. Plaque counts did not increase significantly after the fifth day. When plaques were "picked" and passed either to mice or to monolayers, infection was always produced. In contrast, no infectious organisms could be recovered from areas of monolayers not showing plaques. On microscopic examination, the plaque presented a patch of degeneration and necrosis containing many thousands of crescentic or oval toxoplasmata. The line of demarcation between neutral red stained, uninfected cells and unstained debris was made up of several rows of cells laden with toxoplasmata.

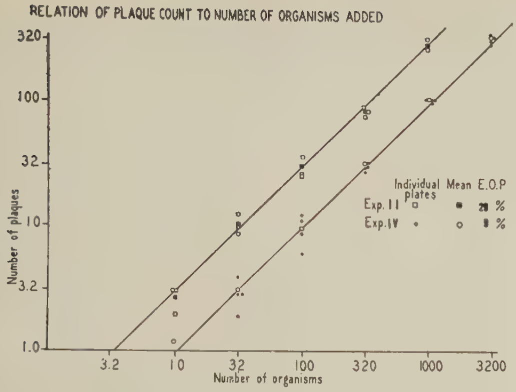

FIG. 1.

Correlation of plaque count with number of organisms plated. Ability to count toxoplasmata directly in a hemocytometer made it easy to calculate the efficiency of plating (E.O.P.) and to demonstrate a linear correlation between number of organisms plated and number of plaques produced. The results of 2 experiments are presented graphically in Fig. 1. That the mean plaque counts fell on straight lines drawn at 45° angles to the abscissa (number of organisms plated) signified that each plaque was induced by infection of a single cell with a single organism(5). In random samples of *Toxoplasma* suspensions, derived from peritoneal exudate of mice (see Materials and methods), the E.O.P. varied between 5.4 and 53% of the counted organisms. An alternate method giving a predictable E.O. P. of about 50% will be described below.

Reproducibility of plaque counts. Degree of reproducibility was tested by replicate plating of 500 parasites on each of 60 monolayers. The suspending medium consisted of 15% EE in Hanks BSS. Thirty plates were overlaid at 90 minutes and the remaining 30 at 180 minutes after infection. Adsorption for 90 or 180 minutes yielded almost identical numbers of plaques. Standard deviations of means for random groups of 5 plates were equivalent to ± 15.8% and ± 14.2%, respectively. Standard deviations from the mean based on counts for 30 *individual* plates were ± 18 or ±18.8%. It was postulated that 95% of replicate samples should give plaque counts reproducible within a range of ± 30 to ± 40% of the mean and that samplings from kinetic experiments were to be considered as comparable if their plaque-forming titers fell within these limits.

Survival of Toxoplasma in various media. Because toxoplasmata die within a few hours in suspending media without added serum or broth(6), a study was made to determine which media were most satisfactory with regard to quantitative survival and E.O.P. Organisms harvested from the peritoneal cavity of an infected mouse were diluted and suspended in various media at a concentration of 2.5 x 10^3/0.5 ml or 1 x 10^3/0.5 ml. These suspensions were incubated at room temperature. Assays for plaque forming units (pfu) were made initially and at 3 to 4, 6, and 24 hours by plating 0.5 ml and adsorbing to the monolayers for 90 minutes before overlaying.

The results of 2 such experiments are summarized in Table I. Plaque counts obtained with freshly prepared suspensions were comparable within the described limits of reproducibility (see above), and therefore loss of viability in subsequent samples was expressed relative to the initial *mean* plaque counts (Table I). Of the media used, Hanks BSS was the least favorable medium, in that 99% of the pfu were lost after 3 hours. In bovine amniotic fluid (BAF), in Earle's BSS containing 0.5% lactalbumin hydrolysate and 0.1% yeast extract (LaYe), and in 5% Ho in Hanks BSS the number of viable organisms decreased by 50% or more in 3-4 hours. On the other hand, media enriched with EE in concentrations of 5 to 50% preserved 80 to 100% of plaque forming activity for at least 3 hours, and 50 to 60% for 6 hours. However, after 24 hours, plaque forming activity had decreased by over 90% regardless of the nature of the medium. All subsequent adsorption studies were made in Hanks BSS containing EE at concentrations from 5-15%.

Adsorption of plaque forming units (pfu). Preliminary data indicated that the same number of plaques was obtained after 90 or 180 minutes of adsorption (see above). More detailed information concerning time required for adsorption of a maximum number of pfu was obtained by overlaying plates at frequent

TABLE I. *In Vitro* Survival of *Toxoplasma* in Different Media at Room Temperature.

		Time of incubation						
		Initial	3-4 hr		6 hr		24 hr	
Exp. No.	Medium	Po	Pt	Pt/Po	Pt	Pt/Po	Pt	Pt/Po
I	Ho20EE5	351	237	.77	—	—	17	.055
	BAF	335	168	.54	—	—	2	.0065
	LaYe	286	122	.39	—	—	2	.0065
	Ho5	299	87	.28	—	—	0	0
	Hanks BSS	276	3	.0097	—	—	0	0
	Initial mean	309						
II	Ho20EE5	135	95	.66	60	.42	2	.014
	EE5	155	127	.89	68	.48	3	.021
	10	162	126	.88	79	.55	5	.035
	15	155	126	.88	74	.52	4	.028
	20	140	138	.97	77	.54	5	.035
	30	141	132	.92	82	.58	—	—
	50	122	148	1.0	85	.59	5	.035
	Hanks BSS	137	2	.014	0	0	0	0
	Initial mean	143						

For description of media see text. Numbers besides ingredients refer to percentages included in Hanks BSS.

Pt = No. of plaques at time "t." Pt/Po = Fraction of initial mean pfu surviving at time "t." — = not tested.

intervals after infection. The results of 6 such experiments are summarized in Fig. 2. In each experiment, plaque counts obtained on plates adsorbed for 70 to 90 minutes or longer were within the limits of reproducibility previously determined for replicate platings of single samples (± 30% of the mean for multiple plates). The mean of all counts falling within these limits was considered as the maximum (P_m). Fig. 2 presents on a log scale the plaque numbers at times t (P_t) relative to P_m. From the resulting average values, fractions of unadsorbed pfu ($1 - P_t/P_m$) were calculated and plotted (Fig. 3). The curve suggested a slow rate of adsorption for the first 20 minutes followed by a more rapid exponential decrease of free pfu between 20 and 80 minutes. The validity of this slope was confirmed by direct assay of unadsorbed organisms in experiment Fig. 4. The velocity constant (Kc) of adsorption between 20 and 80 minutes was calculated from these curves according to the function $P_t/P_m = 1 - e^{-Kct}$ (7) and proved to be Kc = 0.024. This indicated that 9.6×10^{-10} pfu were adsorbed/cell/minute.

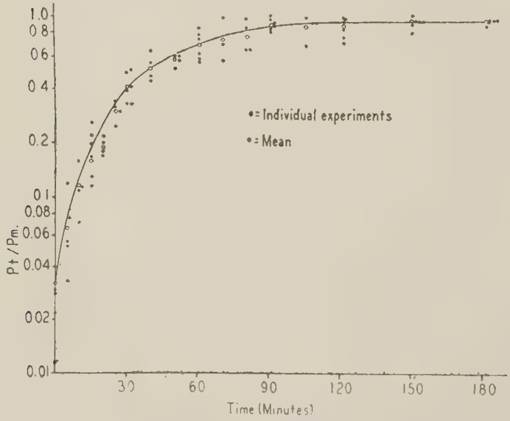

FIG. 2. Increase in No. of plaques with increased period of adsorption.

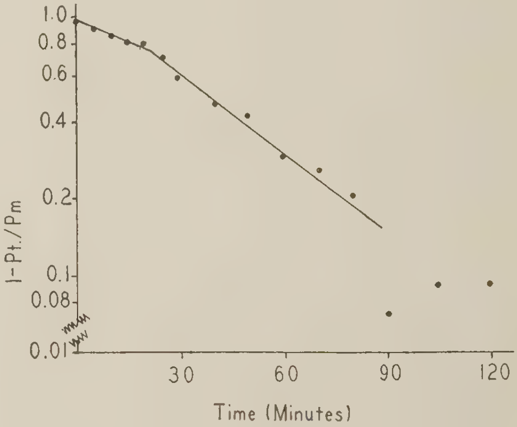

FIG. 3. Decrease of plaque-forming units with increased period of adsorption (calculated from Fig. 2).

Effect of washing, centrifugation, and increased viscosity on adsorption. (a) *Washing.* In several experiments, the effect of

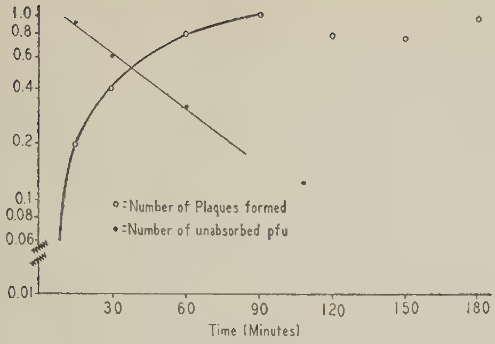

FIG. 4. Experimental determination of slopes for adsorbed and unadsorbed plaque-forming units.

TABLE II. Effect of Viscosity and Centrifugation on Rate of Adsorption of Toxoplasma pfu.

Adsorption time (min.)	Without CMC* No. plaques	Without CMC* Pt/Pm†	With CMC* No. plaques	With CMC* Pt/Pm†
15	22	.05	9	.02
30	128	.29	25	.06
60	271	.62	47	.11
90	396	.91	57	.13
120	423	.97	75	.17
180	492	1.10	83	.19
15 centr.‡	154	.35	91	.21

Inoculum 1,000 toxoplasmata/3.0 ml.

* CMC: 0.4% carboxymethylcellulose (see text).

† Pm (mean 90-180 min.) = 437.

‡ Plates centrifuged at 500 rpm (16 × g) for 15 min. during adsorption. Compare with line 1 (15 min. controls).

washing monolayers before overlaying was studied. The P_m and rate of adsorption of washed plates were identical in each instance with those on unwashed parallel plates. (b) *Increased viscosity and centrifugation.* Rate of adsorption of toxoplasma was significantly slower than that reported for a number of viruses(7,8). Toxoplasmata are unicellular organisms measuring 6 x 2 μ in size and tend to settle out on standing. In this respect, they differ from viruses, and it seemed possible that prolonged incubation might permit spontaneous sedimentation of organisms out of suspension onto the cell layers.[§] This effect should be counteracted by increasing the viscosity of the adsorption medium, intensified by centrifugation during adsorption. Preliminary experiments have confirmed these expectations.

The results shown in Table II were obtained when one group of plates was infected with organisms suspended in the usual medium (15% EE in Hanks BSS). Another group of plates was infected with an aliquot of the same suspension to which 0.4% carboxymethylcellulose (CMC) had been added to increase viscosity of the medium from 1.5 to 13 centipoises. It can be seen that this modification reduced the plaque count at 180 minutes to less than 20% of the Pm which was reached at 90 minutes in the less viscous medium.

As part of the same experiment, some plates, infected in presence or absence of CMC, were centrifuged for 15 minutes at 16 x g and overlaid at the same time as the 15 minute plates which had not been centrifuged. The results (Table II) showed a 7- to 10-fold increase in P_t/P_m in plates subjected to centrifugation.

Although these findings suggested a role of sedimentation in determining the kinetics of adsorption, further work is indicated to separate this effect from that of diffusion, convection(9) and of the spontaneous motility of *Toxoplasma.*

Attempts to standardize E.O.P. As mentioned above, random samples of toxoplasmata harvested from peritoneal exudate of mice had an E.O.P. ranging from 5.4 to 53%. Attempts were made to obtain suspensions yielding consistently high E.O.P. by synchronous lysis of CEF monolayers seeded with enough toxoplasmata in liquid medium to infect each cell. However, less than 1% of the organisms produced in the cells were spontaneously released into the medium at 20 to 100 hours after infection. The cells making up these monolayers were scraped into suspension and lysed artificially as described under Materials and Methods. Under these conditions, fewer than one-third of the liberated organisms were plaque formers.

These findings prompted detailed study of the multiplication of Toxoplasma in CEF monolayers under liquid medium. Cell sheets were exposed to 3 x 10^7, 3 x 10^5, or 3 x 10^3 organisms/ml. The E.O.P. of the infecting suspension was 10%, resulting in effective multiplicities of infection of 1.2 x 10^{-1}, 1.2 x

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[§] The depth of the liquid layer (0.5 ml in a 50 mm Petri dish) during adsorption is approximately 250μ.

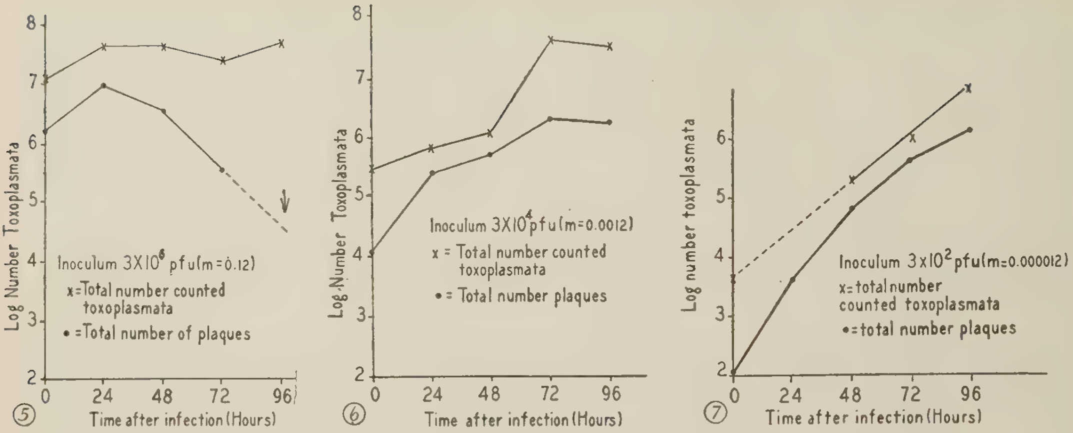

FIG. 5-7. Multiplication of Toxoplasma in chick embryo fibroblast monolayers infected at different multiplicities.

10^{-3}, and 1.2 x 10^{-5} pfu/cell.‖ After 2 hours adsorption, each plate was washed with a total volume of 9 ml of 15% EE in Hanks BSS. The washing fluid was replaced in each plate by 5 ml of medium containing 5% Ho and 15% EE in Hanks BSS. The plates were placed into the CO_2 incubator. At 24 hour intervals, the parasites in the medium and those artificially released from cells were counted and assayed on fresh CEF monolayers.

The results confirmed previous findings that very few toxoplasmata were released spontaneously into the medium. On the other hand, yields of organisms and pfu from artificially lysed cells showed considerable dose dependence. The largest of the 3 inocula (Fig. 5) failed to induce a significant increase in number of counted organisms, although a slightly increased E.O.P. at 24 hours suggested that some multiplication had occurred. At subsequent intervals, the number of organisms remained unchanged, but the E.O.P. decreased progressively, indicating death of the parasites.

In plates infected with 3 x 10^4 pfu (Fig. 6), the parasites multiplied to a peak number of 5 x 10^7 (*i.e.*, average of 2/cell) after 72 hours. At this time, the E.O.P. was only 4.4%, although at 24 and 48 hours it had been around 50%.

The most encouraging results were obtained with monolayers infected at a multiplicity of 1.2 x 10^{-5}/cell (Fig. 7). Here, the number of both parasites and pfu increased exponentially and roughly in parallel up to 96 hours after infection when the experiment was terminated. Under these conditions, high yields of organisms with an E.O.P. of 40 to 50% were obtained.

Subsequent experiments with infection at low multiplicity have confirmed the latter observations so that we have been able to produce at will large populations of toxoplasmata of which about one-half are plaque formers.

Discussion. Infectiousness is the only usable criterion for viability of Toxoplasma. Previous methods of infectivity titrations, whether in animals(10,11) or in mass tissue cultures(11), have given results too variable to provide a suitable basis for quantitative studies on the organism itself. Eyles and Coleman(10) found that one LD_{50} was equivalent to 1 or 30 organisms, depending on whether the suspending fluid was serum-saline or saline solution alone. On the other hand, Chernin and Weller(11) compared the LD_{50} in mice with the ID_{50} in mouse embryo roller cultures, using for inoculation toxoplasmata harvested from tissue cultures. Titers obtained by the 2 methods differed by as much as 100-fold. Only 1 out of 56-540 parasites (1.8-0.19%) was detected by the

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‖ At time of infection, monolayers contained on the average 2.5 x 10^7 cells. Of the seed inoculum of 3.0 x 10^7 cells, 3 x 10^6 were accounted for in the growth medium.

ID_{50} method, and 1 out of 1-36 organisms (100-2.5%) by the LD_{50} method.

In contrast to these methods, the plaque assay is in effect a direct viable cell count (accuracy obtained with only a few plates is at least equal to that obtained by use of a large number of animals in calculating the LD_{50}). In addition, plaques can be detected in 4-5 days, regardless of size of inoculum, whereas mice inoculated with limiting dilutions die only after about 12 days. It has been demonstrated that each plaque is induced by a single parasite, and that large populations of toxoplasmata can be produced at will in which 1 of every 2 organisms is a plaque former.

One problem which has not yet been overcome is our inability to release more than an average of 2 parasites/cell. In view of the fact that chick embryo fibroblasts which line the plaques usually contain a large number of organisms, the low yield under conditions of saturating infections suggests the operation of some yield-limiting mechanism.

The clear definition of individual organisms as infectious units should facilitate further improvement of media with the ultimate aim of supplying the parasite with its needs for extracellular proliferation. Further work is planned leading to purification and biochemical studies of *Toxoplasma*.

Summary. The RH strain of *Toxoplasma gondii* induces formation of necrotic plaques on monolayers of chick embryo fibroblasts overlaid with nutrient agar. Correlation between number of organisms plated and number of plaques produced indicates that each plaque is induced by a single toxoplasma. Survival of toxoplasmata in various media has been tested, and it has been found that Hanks BSS containing chick embryo extract in concentrations of 5 to 50% preserves 80-100% of plaque forming units (pfu) for 3 to 4 hours, 50-60% for 6 hours. Adsorption of pfu on monolayers is maximal after 70 to 90 minutes ($Kc = 0.024$), can be retarded by increased viscosity of the medium, speeded by centrifugation. Artificial lysis of chick embryo fibroblasts infected at low multiplicity yields populations with a predictable efficiency of plating of about 50% of the counted organisms.

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Received August 24, 1959. P.S.E.B.M., 1959, v102.

Assessment of Antral Protective Mechanisms and Intestinal Susceptibility Gradient to Ulcer Formation. (25276)

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Development of peptic ulcer is dependent upon a shift in equilibrium between ulcerogenic factors (secretions of corrosive acid-peptic gastric juices in varying concentrations and volumes), the protective mechanisms (alkaline buffering secretions, *i.e.* bile, pancreatic juice and succus entericus), and bowel susceptibility to ulceration (quantity and character of mucus secretions, mucosal blood supply and regenerative potential, and intrinsic mucosal cellular resistance.) The antral phase of gastric secretion is largely an auto-regulatory system(1) when the antrum occupies its normal position. Potentiation of

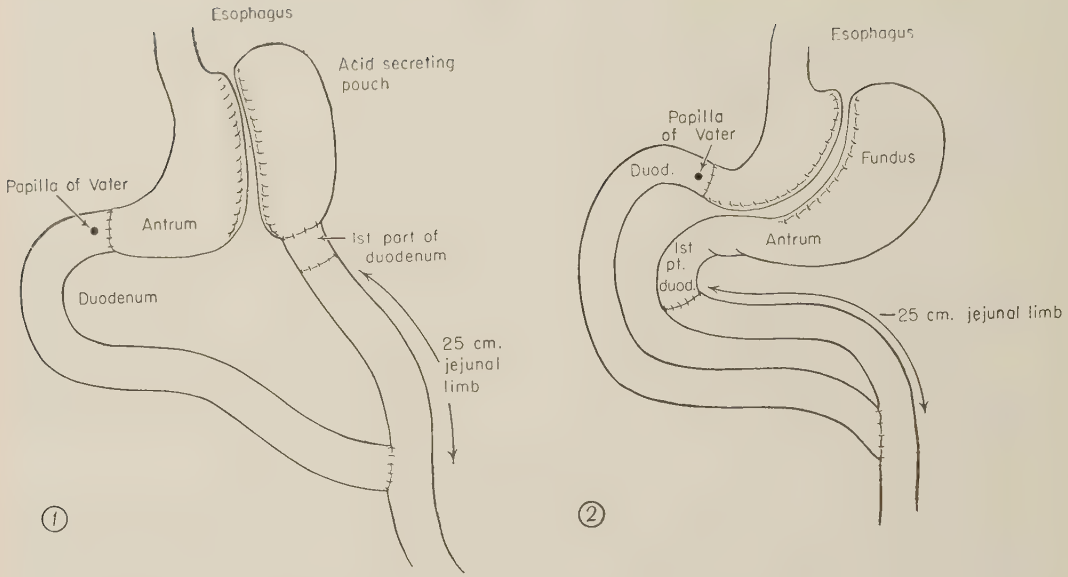

FIG. 1 and 2. Surgical preparation of Group I and Group II animals, respectively.

ulcer production by excluding the antrum from continuity with the proximal acid-peptic secretory area of the stomach is well known (2). A positive gradient of susceptibility to peptic ulceration of the bowel directly related to distance from pylorus was proposed by Mann and Williamson(3). This thesis has been both supported(4) and denied(5). The following preparation was designed for studying protective influences of the antrum and susceptibility gradient to peptic ulceration of the upper small bowel. The preparation also permits assessment of any protection accruing from antral exclusion when acid peptic juice continues to pour over the excluded antrum.

Methods. Two groups of mongrel dogs of both sexes were prepared as follows: (Fig. 1, 2). Procedures were carried out under intravenous nembutal anesthesia (30 mg/kg) and through a midline abdominal incision. In Group I a moderate sized (40%-50%) isolated (denervated) acid secreting gastric pouch was formed as illustrated in Fig. 1. The antrum was left in continuity with the remaining proximal gastric segment. The first part of the duodenum, about 3 cm long, between pylorus and papilla of Vater, was anastomosed isoperistaltically proximally to the isolated gastric segment and distally to a 25 cm Roux-en-Y upper jejunal limb. The distal duodenum containing the papilla of Vater was anastomosed to the antrum. In Group II, (Fig. 2) the stomach was transected obliquely across its longitudinal axis so that the antrum and an equal sized fundic pouch were separated from the proximal gastric segment remaining in continuity with the esophagus. The duodenum was transected about 1 cm proximal to the papilla of Vater. A 25 cm Roux-en-Y upper jejunal limb was anastomosed to the proximal duodenal segment. The distal duodenum containing papilla of Vater was anastomosed to the proximal gastric segment for reconstitution. Postoperatively, the animals were given regular kennel rations supplemented with canned cooked meat daily. Experiment was terminated and all animals sacrificed July 1, 1958. Autop-

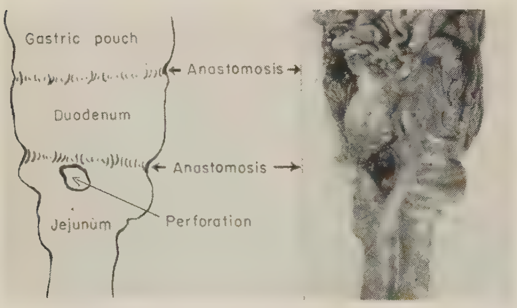

FIG. 3. Example of jejunal perforations occurring in 50% of Group I animals, showing intact proximal duodenal segment.

TABLE I.

	No. of dogs	Survival of sacrificed dogs (mo)		No. of dogs with ulcer	% incidence of ulcer	Site of ulcer	Time of ulcer appearance
		Mean	Range				
Group I	6	8	4-10	3	50%	Jejunum	4, 9, 10 mo
" II	5	6	3- 9.5	0	0		

sies were performed and pertinent results are recorded in Table I.

Results. Of the 6 dogs in Group I, surviving 4-10 months to end of study or dying from peptic ulcer, 3 of them, 50%, developed perforating jejunal ulcers just beyond the site of duodenojejunal anastomosis. (Fig. 3). In none of the animals was the short proximal duodenal segment intervening between the fundic pouch and the jejunum ulcerated or eroded.

In the 5 animals of Group II surviving 3 to 9.5 months, to termination of experiment, there were no erosions or ulcerations in any portion of the bowel. Neither jejunal limb nor duodenal segment showed any evidence of peptic digestion. No evidence of esophagitis was noted in either group. One animal was sacrificed at 3 mo. because of suspected ulcer. No ulcer was found.

Discussion. It is significant that no ulcers were observed in preparation II, in the area of biliary papilla or between it and site of duodenogastric anastomosis (Fig. 2). Presence of antrum in continuity with the acid-secreting fundic pouch in Group II animals undoubtedly accounts for absence of stomal ulcer at this point. The acid secretion bathing the antral mucosa inhibits antral secretion, thereby decreasing secretions of the fundic pouch and the residual proximal gastric segment (auto-regulatory mechanism). Antral mucin and mucus undoubtedly furnish mechanical and chemical protection as well(6). The neutralizing influence of succus entericus of the upper duodenum was a factor common to both groups. Absence of neutralizing effect of bile and pancreatic juice at the neostoma of the isolated pouch was similarly common to both groups.

Upon examination of Group I results, it is evident that both the short upper duodenal segment and the contiguous jejunal Roux limb were exposed to the same acid peptic juice of the isolated pouch. The 50% incidence in this group of a jejunal perforation just distal to the duodenojejunostomy without duodenal involvement suggests definitely a greater susceptibility of the jejunal mucosa to peptic ulceration. The presence of Brunner's glands in this proximal duodenal segment in the dog may partially account for this difference in susceptibility.

This susceptibility gradient has been a controversial subject in the literature. Dillard and Merendino(5) interposed short segments of bowel from various levels between second and third parts of the duodenum to keep constant the alkaline buffering effect of the duodenal contents. Heidenhain or Pavlov pouches were anastomosed to the mid-points of the interposed segments. They failed to produce ulcers in any of these animals and concluded that a susceptibility gradient does not exist.

The phenomenon of decreasing buffering capacity and pH of intestinal contents at levels of small bowel progressively more distal from the papilla of Vater was amply demonstrated by Cross(7).

Summary and conclusions. 1) In Group II type of preparation described herein (antral exclusion with 50% of acid peptic secreting tissue attached to antrum) no abetment of ulcer diathesis was noted. Ulcer was not observed at either neostoma. 2) When, however, a similar fundic pouch was attached to the duodenum (Group I preparation) ulcer occurred at this neostoma in 50% of dogs studied and interestingly not in the short duodenal segment, but just over on the jejunal side, an occurrence which suggests an increased gradient of susceptibility to peptic ulceration.

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Received August 24, 1959. P.S.E.B.M., 1959, v102.

Epinephrine-Induced Alterations in Connective Tissue of Aortic Wall in Rabbits.* (25277)

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Alterations in rabbit aortae caused by epinephrine were first described by Josué(1). Later on several others have confirmed his results. Summaries have been given by Hueper (2) and Raab(3). Gross alterations have been observed as a response to epinephrine injected intravenously once daily through two weeks. The purpose of this experiment was to examine the influence of epinephrine on the connective-tissue ground substance in the aortic wall. The amorphous ground substance is characterized by its content of acid mucopolysaccharides, sulfated as well as nonsulfated, whereas to a considerable degree, the collagen is characterized by its content of hydroxyproline.

Materials and methods. Twenty white male rabbits were kept on a uniform, sufficient laboratory diet. The experimental animals had epinephrine† intravenously injected daily for 15 days in doses rising from 0.025 mg/kg once a day to 0.050 mg/kg twice a day during the last 10 days. On the 14th day experimental animals and controls were injected with ^{35}S sulfate, 1 mC/kg subcutaneously (carrier-free ^{35}S sulfate,‡ diluted to a suitable volume with a 0.005% solution of sodium sulfate in physiological saline). 48 hours later they were sacrificed with nembutal (ethyl-methylbutyl barbituric acid) injected intravenously. The aorta was removed in its whole length together with the heart. The gross alterations were roughly estimated. The thickness of the walls of the right and left heart ventricle was measured. Preparations for histologic examinations were removed from the thoracic and abdominal aorta. The tissues were fixed in a 4% aqueous solution of lead subacetate and a 4% aqueous solution of neutral formalin. The sections were stained with hematoxylin-eosin (Harris), orcein (Unna) and Sudan III. Further the sections were stained with a ½% aqueous solution of toluidine blue for metachromasia, and for calcium by the method of von Kossa. For the biochemical investigations the thoracic aorta from the aortic valve to the diaphragm, the site of the most pronounced gross alterations, was used. The aorta was cut longitudinally into 2 halves, and the intima plus media were peeled off. The water content was determined as the difference in weight before and after 48 hours drying in an oven at 37°C. The total hexosamine content was determined to estimate the content of acid mucopolysaccharides, by the method of Elson and Morgan(4) as modified by Boas(5). The analysis was carried out on half of the dried and minced tissue by hydrolysis with 2 N HCl as described by Kirk and Dyrbye(6). The sulfated mucopolysaccharides were estimated by the *in vivo* uptake of ^{35}S sulfate as devised by Moltke(7). After drying, the uptake was measured on the hydrolysate of the dried tissue, which was also used for the determination of the hexosamine. The activity was measured with a Geiger-Müller tube having a mica-end window weighing 2.7 mg/sq cm. Determination of the hydroxyproline

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* Supported by grants from Reinholdt W. Jorck og Hustrus Fond.

† 0.1% solution of epinephrine hydrochloride.

‡ Obtained from the Radiochemical Centre, Amersham, England.

TABLE I. Gross Alterations in Rabbit Aortae. The water content in % of wet weight, and uptake of ^{35}S sulfate in counts/min./10 mg dried tissue.

Gross alterations*		Water content		^{35}S sulfate uptake	
Exp.	Control	Exp.	Control	Exp.	Control
0	0	71.2	65.5	286	82
4	”	68.1	57.4	322	92
0	”	67.5	69.8	200	124
0	”	72.2	63.8	230	127
2–3	”	68.5	66.7	266	103
1	”	72.7	65.2	155	87
3–4	”	73.2	65.2	204	90
0	”	72.3	62.8	184	90
2	”	69.7	66.3	224	99
2	”	70.2	67.9	140	93
Means		70.6	65.0	221	99
		± 0.7†	± 1.0	± 18	± 5
P value		<.001		<.001	

* Gross alterations were roughly estimated by grading from 0-4.

† Stand. dev. of mean.

should reveal alterations in the amount of collagen. The analysis were made on the remnants of the dried tissue by Neuman and Logan's method(8) as modified by Martin and Axelrod(9).

Results. The gross findings appear from Table I. In 6 out of 10 animals whitish plaques were found scattered chiefly over the thoracic aorta, somewhat less pronounced in the ascending aorta and the arch, and only very few changes could be observed in the abdominal aorta. In one animal the whole thoracic aorta was altered. The lesions brought about a thinning of the wall and a few aneurysms. Thickening of the walls of the heart ventricles was found mainly on the left side. These changes were most pronounced in the animals exhibiting the most marked alterations of the aorta. Furthermore several animals showed effusions in the serous cavities, particularly the pleura and the pericardium.

Microscopically, scattered necrotic areas of media with calcium deposits were found, and in their periphery, accumulations of amorphous ground substance, which stained metachromatically with toluidine blue indicating a content of acid mucopolysaccharide(10). A moderate proliferation of fibroblasts and a fragmentation of the elastic membranes were observed. Microscopic alterations were found also outside the grossly visible lesions. By fat-staining of frozen sections no difference between experimental animals and controls was noted, particularly no deposition of fat near the media lesions. In a few animals a definite proliferation of the intima was observed, especially in areas presenting media necrosis.

Table I shows, that the content of water was somewhat higher in the aortae of the experimental animals than in the controls. The hexosamine values (Table II) showed a significant rise in the experimental animals, whereas the hydroxyproline values were somewhat lower than in the controls, but the difference was not significant. Accordingly the hexosamine/hydroxyproline ratio (Table II) was higher for the experimental animals. The uptake of ^{35}S sulfate is listed in Table I. It appears that the uptake in the experimental animals was considerably greater than in the controls.

Discussion. The observations of gross and microscopic alterations in aortae accord with previous investigations. The increased uptake of ^{35}S sulfate and the increased water content together with the findings of metachromasia of the ground substance and the hexosamine increase suggest an accumulation of acid mucopolysaccharide. 24-48 hours after peritoneal injection the main part of ^{35}S sulfate is likely to be found esterified in chondroitin sulfuric acid in different tissues, among others the aorta(11). Among the acid mucopolysaccharides, hyaluronic acid has a

TABLE II. Content of Hexosamine and Hydroxyproline in Rabbit Aortae, μg/mg Dried Tissue and the Hexosamine/Hydroxyproline Ratio.

He		Hp		He/Hp	
Exp.	Control	Exp.	Control	Exp.	Control
6.7		14.2	15.4	.47	
7.2	7.1	15.1	19.5	.48	.36
8.5	6.9	13.2	14.6	.64	.47
8.6	6.4	18.3	16.6	.47	.39
7.0	6.4	15.2	18.1	.46	.35
6.1	6.0	17.3	20.2	.35	.30
7.4	6.1	16.3	23.4	.45	.26
7.4	4.8	21.7	19.2	.34	.25
6.7	5.3	15.9	14.5	.42	.37
6.4	5.7	16.8	20.7	.48	.28
7.2	6.1	16.4	18.2	.45	.34
±.2*	±.2	±.8	± 1.0	±.03	±.02
		P value			
<.01		<.10		<.01	

* Mean ± stand. dev. of mean.

considerable water-binding capacity. In human aortae the greater part of the mucopolysaccharide fraction is chondroitin sulfuric acid but other mucopolysaccharides including hyaluronic acid seem to be present(12). While the hydroxyproline content showed no definite change, there was an increase in the hexosamine/hydroxyproline ratio indicating an increase in the amount of amorphous ground substance in proportion to the collagen.

The mechanism of the epinephrine effect is not clear. Theories have been advanced of vascular injury as a result of the variations in blood pressure accompanying the epinephrine injections; of an anoxic injury via the vasa vasorum; and of a direct effect on the cellular metabolism. The accumulations of acid mucopolysaccharides noted in these experiments is probably a reflection of reparative processes started by the epinephrine induced injury. Healing wounds contain large amounts of acid mucopolysaccharides, and the increased uptake of ^{35}S sulfate accord with previous observations of wound healing (13,14).

Summary. Alterations in rabbit aortae were brought about by intravenous administration of epinephrine. Microscopic examination revealed accumulation of metachromatic ground substance. The aortic content of hexosamine and water together with the uptake of ^{35}S sulfate was increased, whereas the amount of hydroxyproline was unaltered. The increase in the mucopolysaccharide/collagen ratio may indicate a reparative process.

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Received August 24, 1959. P.S.E.B.M., 1959, v102.

Local Morphological Response Following a Single Subcutaneous Injection of Carrageenin in the Rat. (25278)

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Various polysaccharides, mostly of bacterial origin, can induce inflammatory conditions in experimental animals(1,2,3,4). Subcutaneous injections of carrageenin, a polygalactose-sulfate of plant origin, have been used in different species for biochemical analysis of collagen formation(5,6,7,8). The present study of morphological response to a single subcutaneous injection of carrageenin in the rat was carried out to determine whether this plant material could be used as a stimulus to induce an inflammatory lesion.

Material and methods. Seven groups of 6 female Sherman rats (body-weight range 140 ± 10 g) were housed individually and fed Purina Laboratory Chow and tap water *ad lib.* All animals received a single subcutaneous injection of 0.5 ml of a 2% aqueous solution of carrageenin† (molecular weight 100,000 to 500,000 g/mol), on mid-line of back, halfway

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† Obtained through courtesy of Mr. Leonard Stoloff, Seaplant Corp., New Bedford, Mass.

between tail root and thorax. Groups of animals were sacrificed by chloroform after 1st, 2nd, 4th, 7th, 14th, 21st, and 28th day; and grossly recognizable abnormal structures of subcutaneous tissues were dissected and weighed on a Roller-Smith torsion balance. Dissected specimens were fixed in formalin-alcohol (1:9 V/V), and paraffin sections were stained with Masson's trichrome stain, the Alcian blue method for acid mucopolysaccharides, and with periodic-acid-Schiff reagent (PAS). Selected sections of each group were stained with Toluidine blue for metachromasia, and with Gomori's technic for reticulin fibers. In an additional weight study 5 groups of 12 animals were injected and sacrificed after 1st, 2nd, 4th, 7th, and 14th day. Grossly recognizable structures were removed, weighed on a Roller-Smith torsion balance, placed in tared vials, and dried to constant weight at 105 ± 5°C. Dry weights of residues were determined, and weights of volatile matter were obtained by difference.

Results. Immediately after administration of carrageenin, localized swelling of approximately 15 mm in diameter at site of injection could be noted which disappeared within an hour. All animals showed normal behavior, and gained weight. Gross and microscopic findings are described after various time intervals.

First Day—Gross findings. After removal of skin a slightly yellowish-white layer of very soft gelatinous consistency, poorly defined, was found in area from axillary to inguinal region. There was a slight hyperemia of surrounding tissues. *Microscopy.* Large areas of exudate with metachromatic staining properties and a rather weak positive reaction after Alcian blue stain indicated presence of carrageenin. Numerous ruptured collagen fibers which stained faintly blue with the trichrome method were found within this exudate, together with a large number of polynuclear and mononuclear leukocytes, most of them in diffuse distribution, but focal accumulation also occurred rather frequently. Erythrocytes were less commonly found, although in some instances parts of exudate were hemorrhagic. Occasionally, macrophages showed phagocytosis of carrageenin as shown by intensive intracellular metachromasia and positive, blue-green Alcian blue stain. Precipitation of fibrin was a rather common finding.

Second Day—Gross findings. Appearance of layer was almost identical with the findings after the 1st day with the exception that the lesion was less gelatinous. *Microscopy.* The findings were very similar to those described after the 1st day with the exception that the number of carrageenin-containing macrophages had increased and the uptake of polysaccharide material was more marked.

Fourth Day—Gross findings. A slightly yellowish-white layer of compact but still soft consistency was spread over a considerable area of the flank, and was poorly defined. There was a slight hyperemia of surrounding tissues. *Microscopy.* The large amount of stainable exudate had all but disappeared; only remnants were found. Their staining properties were essentially the same as described after the 1st day, indicating that carrageenin was still present. The major portion of carrageenin was found in numerous macrophages of foam-cell-like appearance after trichrome stain. Plasma of these cells showed a marked positive periodic-acid-Schiff reaction, strong metachromasia and a blue color after staining with Alcian blue. Numerous fibroblasts were intimately mixed with these macrophages. Multiple focal accumulations of leukocytes were found which often contained central necrotic areas consisting of cellular debris, fibrin, and ruptured collagen fibers.

Seventh Day—Gross findings. A thin whitish-pink layer of rather dense fibrous-like tissue was spread over a considerable area of the flank and was poorly delineated. No vascular reaction of surrounding tissues was visible. Pre-existing connective tissue could not easily be distinguished from the induced structures. *Microscopy.* Numerous fibroblasts, young capillaries and carrageenin-containing macrophages were found, forming granulation tissue which had walled off one or multiple necrotic areas. These necroses contained cellular debris, fibrin, and amorphous protein. Most macrophages at this time maintained their foam-cell-like appearance after trichrome stain and Alcian blue method, but after the periodic-acid-Schiff method and the

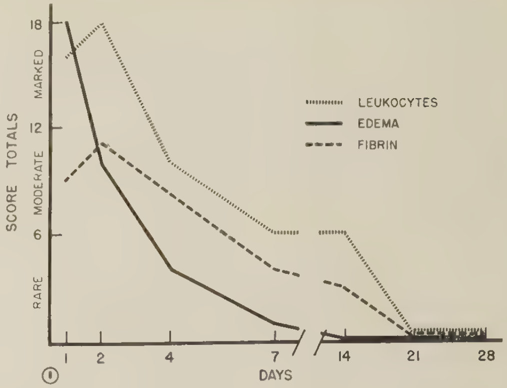
Toluidine blue stain the phagocytized polysaccharide appeared as multiple small droplets. Curly strands of collagen fibers and a fine network of reticulin fibers were found between the cellular elements. Some apparently newly-formed fat cells contained very small amounts of polysaccharide material which could be seen as little globules in a barely stainable cytoplasm. These were attached to certain parts of the cell border. The rest of the cell was occupied by a large optically empty area, which indicated former presence of fat.

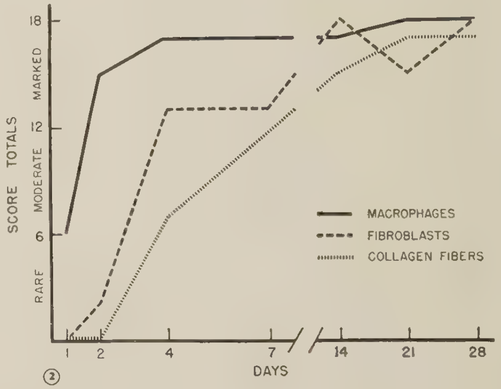
Fourteenth Day—Gross findings and microscopy were similar to observations after 7th day.

Twenty-first Day—Gross findings. There was a thin whitish-gray layer of compact fibrous tissue spread over a smaller area on the flank. The structure was not easily distinguished from surrounding tissues. *Microscopy.* Almost no necroses were found at this stage. In several areas of previously described granulation tissue, a decrease of number of macrophages, fibroblasts, and capillaries was found together with a marked fibrosis, *i.e.*, new formation of collagenous and argyrophilic fibers. Other parts of the granuloma still showed numerous macrophages containing polysaccharide material (positive reaction with PAS, Toluidine blue, and Alcian blue) and fibroblasts which were intimately mixed and packed in a firm meshwork of reticulin fibers.

Twenty-eighth Day—Gross findings and microscopy were similar to observations after the 21st day. An attempt was made to obtain a crude quantitative estimate of different characteristic tissue components of the removed structures after different time intervals.

All slides were examined and the following changes were graded: edema, polynuclear leukocytes, fibrin, macrophages, fibroblasts, and formation of collagen fibers. Grading was done by assigning 0 for absent, 1 for rare, 2 for moderate, and 3 for marked occurrence. Since 6 specimens of 6 different animals were used, the evaluation was based upon the following score totals: 0 to 6 = rare; 6 to 12 = moderate; and 12 to 18 = marked. The results are shown in Figs. 1 and 2.





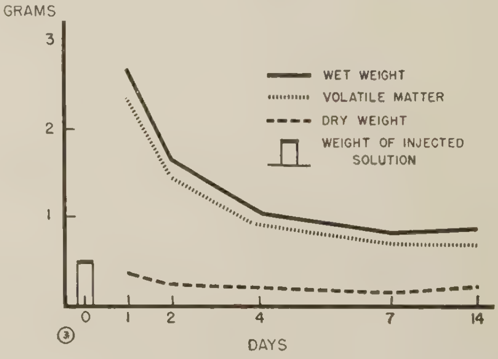


FIG. 1. Score totals for leukocytes, edema, and fibrin of carrageenin-induced lesions, 6 rats/point, at different time intervals after inj.

FIG. 2. Score totals for macrophages, fibroblasts, and collagen fibers of carrageenin-induced lesions, 6 rats/point, at different time intervals after inj.

FIG. 3. Weight relations of carrageenin-induced lesions at different time intervals, 12 rats/point.

These morphological studies show that a single subcutaneous injection of 0.5 ml of a 2% aqueous solution of carrageenin in rats caused a localized response which could be divided easily into 2 stages. The initial phase (1st to 4th day) was characterized by one or more central necrotic foci, surrounded by exudate with leukocytic infiltration, precipitated fibrin, slight hemorrhage and a moderate proliferation of macrophages containing carrageenin. These findings were interpreted as an acute localized inflammatory response in the form of an abscess.

After 4th day, the abscess was walled off; debris and exudate gradually disappeared and were replaced by proliferating macrophages and fibroblasts and by formation of new fibers and capillaries. Thus, the abscess was transformed into a granuloma in the process of repair with ultimate scar formation.

To quantitate the amount of initial inflammatory exudate, *i.e.*, fluid, an additional weight study was carried out as described under Material and methods. Relationships between wet weight, dry weight, and weight of volatile matter of the excised lesions at various intervals up to 2 weeks are shown in Fig. 3.

Discussion. So far only Williams(9) has furnished information about the morphological findings at different time intervals after a single subcutaneous injection of carrageenin in the guinea pig. His observations, however, differ somewhat from our findings in the rat, since he did not find fibrin or fibrinoid material, and only a moderate polymorphonuclear leukocytic response in early stages of the induced lesions. Our observations in the rat indicate a marked leukocytic response and a moderate precipitation of fibrin in the lesion as a common finding during the 1st to 4th day. Species differences may also account for the difference in maximum wet weight of removable srtuctures. In the guinea pig, Robertson and Schwartz(5) reported a maximum at 14th day after a single subcutaneous injection of 16.6 mg of carrageenin/100 g body weight, and Jackson(7) found a maximum at the 7th day after 7.7 mg/100 g body weight. In the rat we observed a maximum wet weight after 24 hours using 7.2 mg of carrageenin/100 g body weight.

All these differences indicate that carrageenin stimulates a more violent acute inflammatory response in the rat, whereas the guinea pig reacts in a more chronic way by forming excessive granulomatous structures.

R. Meier *et al.*(3) used another polygalactoside, namely 5 mg of purified agar/105 g body weight in rats, and observed only a very weak reaction by the wet weight (30 mg) of lesions removed after 7 days. Although we have used larger animals (140 g) and a larger amount of polygalactoside (7.2 mg), our mean wet weight after 7 days was still 920 mg, indicating a more violent and extensive response.

Whether this marked difference is due to high degree of sulfation, approximately 23% of carrageenin in comparison with 1.3 to 1.4% of agar(10) remains to be elucidated.

These studies show that carrageenin, administered as a single subcutaneous injection in the rat, is an irritant which produces a local response. This reaction, a well-defined accumulation of exudate containing fibrin, polynuclear leukocytes, and erythrocytes is, by definition of Boyd(11), inflammation.

Summary. 1. A single subcutaneous injection of carrageenin in rats caused a local tissue response, which from the 1st through the 4th day could be defined as an inflammatory lesion in the form of an acute abscess, which later (5th through the 28th day) was gradually transformed into a polysaccharide-containing granuloma. 2. Weight data indicate a maximum exudative response after 24 hours.

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Received August 24, 1959. P.S.E.B.M., 1959, v102.

Studies Using Anterior Pituitary Hormones as Antigens.* (25279)

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Production of antibodies to anterior pituitary hormones became a subject of controversy following the report of Collip and Anderson that animals receiving repeated injections of TSH and gonadotrophin became refractory to these hormones(1,2). The results of subsequent work on this subject were conflicting; even where evidence of antibody formation was obtained, there remained the possibility that such antibodies were not to the hormone itself, but rather to impurities in the hormone preparations(3-7). More recently, Cruickshank and Currie reported on the production of precipitating antibodies to human growth hormone, ACTH and TSH, which were not, however, hormone specific (8). In the past year, reports have appeared which present evidence for antigenicity of growth hormone(9-12). Thus, Read has reported on production of hemagglutinating antibodies to human growth hormone, and applied anti-human growth hormone sera to detection of growth hormone in human body fluids(10). Hayashida and Li produced precipitating antibodies to bovine and human growth hormones which could inhibit the effects of growth hormone on the epiphyseal cartilage of the hypophysectomized rat (11,12).

Methods. In our laboratory, thyroidectomized rabbits were immunized against bovine TSH, adrenalectomized rabbits against porcine ACTH, and intact rabbits against bovine, porcine and human growth hormones. For immunization, these hormones were emulsified in Freund's adjuvant (complete), and also precipitated with alum. Total dosage of each hormone varied from 5 to 80 mg, and a variety of immunization schedules and routes were used. At suitable intervals the rabbits were bled and sera tested for antibodies using a hemagglutination test in which antigen is coupled to red blood cells by means of bis-diazotized benzidine(13-16). In this test, hemagglutination occurs when antigen-sensitized red cells are suspended in antiserum, and hemagglutination is inhibited by preincubating the antiserum with specific antigen. Using this technic, the following determinations were carried out on each antiserum: 1) titre, 2) specificity, and 3) smallest quantity of specific antigen which could be detected in the system by means of hemagglutination inhibition (Table I).

Results. Antibodies in good titre were obtained to all substances used as antigens. With TSH, extensive cross reactions occurred, although much smaller quantities of TSH (0.1 μg) were required to consistently produce hemagglutination-inhibition of TSH antiserum as compared with other pituitary hormones (10 μg). These cross reactions are not surprising since the TSH which we used was relatively impure.

From the studies done, the antibodies to ACTH appeared to be hormone specific but not species specific. Thus either porcine or human ACTH in quantities as little as 0.01 μg consistently produced hemagglutination inhibition.

ACTH preparations from a number of animal species are known to be biologically active in man and other animals. Lack of species specificity of ACTH antiserum which we have demonstrated is in keeping with the known biological activity of ACTH from various animal species.

Antibodies to human growth hormone were for the most part hormone specific, although some cross reactions with human ACTH did occur. Whereas as little as 0.01 μg of human growth hormone consistently produced hemagglutination-inhibition, at least 1 μg of human ACTH was required for this reaction.

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* This work supported by grant-in-aid of Nat. Research Council, Canada.

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TABLE I.

Antigen*	Antibody titer†	Hemagglutination-inhibition (μg)‡									
		BGH	PGH	HGH	P ACTH	H ACTH	PGT	BTSH	PROL	BGG	HGG
BGH	1/8000-1/256,000	1	0	0	0	ND	0	0	ND	1	ND
PGH	1/80 -1/1280	0	.01	0	0	"	0	0	"	ND	"
HGH	1/64 -1/12,800	0	0	.01	0	1	0	0	0	"	0
P ACTH	1/20 -1/8000	0	0	0	.01	.01	0	0	0	"	ND
BTSH	1/1000-1/8000	10	10	ND	10	ND	10	.1	ND	0	"

* BGH, bovine growth hormone (Frank W. Horner); PGH, porcine growth hormone (Nordic Biochemicals); HGH, human growth hormone (supplied by Dr. Phillip Henneman); all growth hormone prepared by Raben technic; P ACTH, porcine adrenocorticotrophic hormone (Nordic Biochemicals); H ACTH, human adrenocorticotrophic hormone (Nordic Biochemicals); PGT, porcine pituitary gonadotrophin (Ferring Ab. Malmo); BTSH, bovine thyroid stimulating hormone (Nordic Biochemicals); PROL, porcine prolactin (Ferring Ab. Malmo); BGG, bovine gamma globulin; HGG, human gamma globulin.

† Range of titer of antisera from a group of animals.

‡ Where a figure is given, this represents smallest quantity of antigen which would consistently inhibit hemagglutination.

0 = no hemagglutination inhibition; ND = not done.

Cross reactions between anti-human growth hormone sera and ACTH were also reported by Read(10).

Antibodies to bovine growth hormone showed cross reactions with bovine gamma globulin, which could be eliminated by prior absorption of the serum with bovine gamma globulin. This is consistent with the findings of Hayashida and Li(11). Antibodies to porcine growth hormone appeared from the studies done, to be hormone specific.

Growth hormone antisera showed no cross reactions with growth hormone preparations from other species. This may be interpreted as immunologic evidence for species specificity of growth hormone, a property which is well known biologically(17), and which is consistent with the report of Hayashida and Li (12).

Read reported on the use of anti-human growth hormone sera in assaying growth hormone in human body fluids(10). In our laboratory the application of human growth hormone and ACTH antisera to detection and measurement of these hormones in human body fluids is in progress.

Summary. Evidence is presented for antigenicity of TSH, ACTH, and growth hormone. Rabbit antisera to these hormones were prepared and then tested by means of a hemagglutination test in which antigen is coupled to red blood cells, by means of bis-diazotized benzidine. The antibodies to TSH showed extensive cross reactions with other pituitary hormones. The antibodies to ACTH appeared to be hormone specific but not species specific. Antibodies to bovine, porcine, and human growth hormones appeared to be species specific, and for the most part, hormone specific. The application of human growth hormone and ACTH antisera to detection and measurement of these hormones in human body fluids is in progress.

The adrenalectomies were kindly performed by Dr. J. R. McCorriston.

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Received August 24, 1959. P.S.E.B.M., 1959, 102.

Serum and Liver Lipids in Rats Treated with Neomycin. (25280)

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Samuel *et al.*(1) reported recently that oral administration of neomycin was associated with significant decrease in serum cholesterol concentration in ten patients studied. Neomycin, an antibiotic isolated from *Streptomyces fradiae* by Waksman and Lechevalier, (2), is effective *in vitro* and *in vivo* against a wide variety of bacteria including species resistant to other antibiotics(3). This antibiotic seems to have a large use in veterinarian medicine(4) and probably will gain in importance in handling the infections of laboratory animals in research institutions. The possible effect of neomycin on lipid metabolism of experimental animals may become, however, an undesirable complication in some studies. Because previous data on this subject concern only human material(1), it seemed interesting to investigate the effect of neomycin on lipids in various laboratory animals. This report deals with data on serum and liver lipids in rats fed during 4 months with normal diets and cholesterol rich diets, alone or with neomycin.

Method. Male Sprague-Dawley rats, weighing 172-187 g, were used. The basal control diet was a grain food of the following composition(5): ground yellow corn 68%; linseed oil meal 13%; powdered skim milk 15%; alfalfa leaf meal 2%; cod liver oil 1%; iodized table salt 0.5% and calcium phosphate 0.5%. Basal diet supplemented with 4% cholesterol in 5% of olive oil was called "cholesterol diet." When basal diet or cholesterol diet was added with 0.2% neomycin (Mycifradin Sulphate, Upjohn Co.), the diets were called, respectively, "neomycin diet" and "neomycin + cholesterol diet." Dosage of neomycin was arbitrary. Samuel *et al.*(1) gave their patients 0.5 to 2 g of Mycifradin Sulphate daily. In our experiment a rat ingested approx. 30 mg daily of same preparation, which was a large intake as compared with dosage in humans. Total cholesterol of serum and liver was determined following the method of Abell *et al.*(6). For study of lipid phosphate samples were extracted with alcohol-ether and analysis carried out by the technic of Zilversmit and Davis(7).

Results. Tables I and II summarize data on the effect of various experimental diets, on lipid levels in serum and liver of rats. Despite the slight differences in average values noted between the 4 groups of animals, no marked effect of neomycin on phospholipids or total cholesterol levels in serum and liver was observed. Yet, in this study a relatively large dosage of neomycin was used over a prolonged period.

Samuel *et al.*(1), in their comment on post-neomycin hypocholesterolemia in man, mentioned the possibility that this effect might be due to changes in bacterial flora or certain enzyme systems of the gastro-intestinal tract. This might be true as far as the human subject is concerned. In rats, however, neomycin

TABLE I. Serum Lipids (mg/100 ml) in Rats after Feeding with Various Diets during 4 Month Period. Mean, S.D. and range.

Diet	No. of rats	Final body wt (g)	Total cholesterol	Phospholipids	C/P*
Normal control	20	345 ± 37 281-408	67 ± 17 36-104	130 ± 14 100-150	.52 ± .12 .25-.71
Neomycin	13	318 ± 31 272-395	83 ± 16 57-105	139 ± 22 108-183	.61 ± .15 .42-.81
Neomycin + cholesterol	19	314 ± 26 261-361	85 ± 25 42-145	160 ± 25 90-223	.56 ± .19 .22-1.01
Cholesterol	20	385 ± 31 349-427	110 ± 31 65-203	133 ± 26 103-195	.66 ± .90 .41-1.73

* Cholesterol/phospholipids ratio,

TABLE II. Liver Lipids (mg/100 g Wet Tissue) in Rats after Feeding with Various Diets during a 4 Month Period. Mean, S.D. and range.

Diet	No. of rats	Liver wt (g)	Total cholesterol	Phospholipids	C/P
Normal control	20	11.4 ± 1.8 8.3-14.1	252 ± 42 202-348	2849 ± 252 2440-3350	.09 ± .02 .07-.16
Neomycin	15	10.1 ± 1.5 7.6-12.1	295 ± 45 225-321	2912 ± 218 2400-3398	.09 ± .03 .08-.14
Neomycin + cholesterol	18	10.2 ± 1.3 7.6-12.8	1660 ± 350 1026-2333	3017 ± 388 2300-3725	.53 ± .14 .36-.89
Cholesterol	20	15.0 ± 2.7 13.5-16.1	1676 ± 266 1260-2043	2880 ± 413 2020-3675	.61 ± .13 .38-.95

did not influence lipid levels in serum and liver under the described experimental conditions.

It was believed for a long time that rats were resistant to induction of hyperlipemia and hypercholesterolemia. Recently, however, this opinion has been modified(8) and these animals are now frequently used for study of various dietary factors inducing atherosclerosis.

In our experiment no significant hypercholesterolemia was observed, despite the prolonged ingestion of cholesterol rich diet by rats.

The only significant change in this study was the rise of total cholesterol in livers of animals given cholesterol rich diets, with and without neomycin.

Summary. 1) Because ingestion of neomycin results in hypocholesterolemia in human subjects, the effect of this antibiotic on lipids of serum and liver has been studied in rats. 2) Basal control diet or cholesterol rich diet, alone or added with 0.2% neomycin, has been fed to rats for 4 months. This prolonged administration of neomycin to rats was not associated with any significant changes in serum or liver levels of cholesterol and phospholipids.

The author is indebted to Upjohn Co. of Canada for generous supply of Mycifradin Sulphate.

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Received August 28, 1959. P.S.E.B.M., 1959, v102.

Comparison of Glutamic-Oxalacetic and Glutamic-Pyruvic Transaminase Concentrations in Human Saliva and Serum.* (25281)

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Since development of practical methods for determination of glutamic-oxalacetic and glutamic-pyruvic transaminases, measurements of serum and cerebrospinal fluid concentrations of these enzymes have been used in differential diagnosis of an ever increasing list of disease states. These enzymes are present in varying amounts in all body tissues and function in the transference of amino groups from one amino acid to another. Increased

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* Northwestern University studies in nutrition at Jefferson-Hillman Hospital, Birmingham, Ala. These studies supported by grants from Roche Anniversary Foundation and Pfizer Fn.

TABLE I. Comparison of GOT and GPT Content in Saliva and Serum of 81 Patients.

Group	No.	Mean		σ*		r†	σ_r‡	r/σ_r	Incubated saliva	
		Saliva	Serum	Saliva	Serum				Mean	σ
Glutamic-oxalacetic transaminase—units/ml										
Males	33	28.7	16.3	24.4	8.6	–.066	.173	.38	2.0	4.4
Females	48	26.9	20.2	15.9	9.2	.341	.129	2.64	4.4	8.1
Total	81	27.5	18.6	20.2	9.2	.116	.110	1.06	3.4	7.0
Edentulous	39	24.9	19.0	20.9	10.1	.241	.151	1.60	3.6	7.7
Dentulous	42	30.2	18.2	19.9	8.3	–.061	.154	.40	3.3	6.2
Glutamic pyruvic transaminase—units/ml										
Males	33	11.9	15.9	9.0	9.9	.292	.159	1.84	158.2	104.1
Females	48	12.9	16.8	10.4	10.6	.080	.140	.57	173.5	105.9
Total	81	12.5	16.4	9.9	10.2	.163	.108	1.51	167.3	107.0
Edentulous	39	9.2	17.2	7.1	11.7	.144	.157	.92	153.6	111.4
Dentulous	42	15.5	15.7	11.1	8.6	.282	.142	1.99	180.0	100.3

* σ = Stand. dev. † r = Coef. of correlation. ‡ σ_r = Stand. error of coef. of correlation.

levels in blood reflect damage to those organs or systems which are particularly rich in these enzymes. Human saliva contains detectable amounts of many enzymes found in blood(1). Existence of transaminases in saliva has recently been reported(2). The present studies were undertaken to determine levels of GOT and GPT in human saliva and to compare concentrations contained therein with those of concurrently collected samples of serum.

Materials and methods. Paraffin stimulated whole saliva and venous blood were obtained from 81 consecutive outpatients of the Nutrition Clinic, Hillman Hospital. The 81 subjects ranged from 12 to 79 years and consisted of 27 white males, 6 negro males, 39 white females and 9 negro females. Thirty-nine were edentulous and 42 had some or all of their natural teeth. Fifteen ml of saliva and 5 ml of blood were collected concurrently from each patient. Blood was centrifuged immediately following collection. Serum was drawn off and stored in refrigerator with saliva samples until ready for testing. The GOT and GPT content in units/ml was determined in 0.2 ml aliquots of each specimen of saliva and serum by the colorimetric method of Mohun and Cook(3). Readings of per cent transmittance were made in a Coleman Universal spectrophotometer set at wave length of 550 mμ. Since many bacteria contain GOT and GPT (4), 10 ml of each saliva sample were added to sterile brown screw cap 25 ml bottles containing 100 mg dextrose. The bottles were sealed, incubated and agitated in water bath maintained at 37°C for 4 hours. Ten ml of pooled serum treated in identical fashion served as control. After 4 hours, 0.2 ml of each incubated aliquot was analyzed for GOT and GPT by above method. The data were then subjected to statistical analysis.

Results. Each saliva sample contained measurable quantities of GOT in 5 to 109 units/ml. GPT was found in 72 of the 81 (88.9%) saliva samples in amounts ranging from 2 to 43 units/ml. The saliva GOT content exceeded that of GPT in 74 of the 81 (91.4%) cases. Incubation of saliva with glucose at 37°C for 4 hours resulted in each instance in a pronounced increase in GPT concentration and decrease in GOT content. In contrast, incubation of pooled serum with glucose did not materially alter the GPT level but was accompanied by a significant depression in GOT value. The serum GOT and GPT concentrations in each patient were within the generally accepted limits of normal, none exceeding 39 units/ml.

The mean GOT and GPT concentrations in serum and saliva of the 81 patients and the statistical evaluation are shown in Table I. Mean GOT levels were: serum, 18.6; saliva, 27.5; and saliva incubated with glucose, 3.4 units/ml. The corresponding mean GPT concentrations were: 16.4, 12.5, and 167.3 units/ml respectively. There was no significant correlation between the serum and saliva levels of these enzymes, the correlation coefficients (r) being 0.116 for GOT and 0.163 for GPT (Fig. 1). Neither of these values exceeded

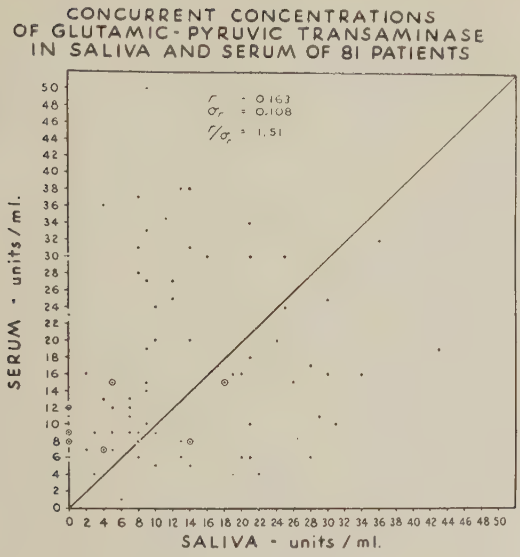

FIG. 1.

the standard error of coefficient of correlation (σ_r) by the 3 or more times required for statistical significance.

There was no appreciable sex difference in mean serum and saliva levels of GOT and GPT (Table I). The edentulous patients, as a group, had lower levels of both GOT and GPT in saliva than did the group with some or all of their natural teeth. A similar finding obtained for GPT but not for GOT when saliva was incubated with glucose. The difference between mean saliva GPT values in dentulous and edentulous patients was statistically significant, the actual difference between means being 3.07 times greater than the standard error of difference between means (σ_D). By the same formulation, the differences in mean levels of GPT in incubated saliva-glucose mixtures and of GOT in saliva and in incubated saliva-glucose mixtures were not significant.

Discussion. These studies demonstrate that GOT and GPT are common constituents of human whole saliva. In the absence of pathologic conditions associated with elevated blood transaminase concentrations, the mean GOT content of saliva exceeds that of serum and mean serum GPT content surpasses that of saliva. Lack of a significant correlation between serum and saliva values indicates that the concentration in saliva is not a direct reflection of that in blood.

The present findings suggest that the oral flora and oral tissues both contribute to transaminase content of saliva. Incubation of whole saliva with glucose, which stimulates growth of oral glycolytic microorganisms was followed in each instance by a striking elevation in the saliva GPT level and the almost complete disappearance of GOT from samples so treated. The rise occurred in aliquots from each sample of saliva including those which did not contain measurable amounts of GPT before incubation. The significantly higher saliva GPT value in the group of patients with natural teeth in contrast to the edentulous group provides evidence that the natural attrition of the supporting structures of the teeth and superimposed destructive processes affect the transaminase content of saliva. The degree to which the transaminase concentration of saliva is affected by oral and periodontal disease remains to be determined.

Summary and conclusions. Detectable quantities of GOT and GPT were found in 100% and in 89% respectively of samples of stimulated saliva from 81 patients. The saliva GOT content exceeded saliva GPT level in 91% of cases. Incubation of an aliquot of each saliva sample with glucose resulted in a pronounced increase in GPT and diminution in GOT. Comparison of saliva transaminase levels with those of concurrently collected samples of serum showed no significant correlation, thus signifying that the prime and immediate source of saliva transaminase is oral rather than systemic.

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Received August 25, 1959. P.S.E.B.M., 1959, v102.

Hypotensive Action of Progesterone in Experimental and Human Hypertension. (25282)

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Considerable evidence has accrued to suggest that disturbed electrolyte metabolism may be one of the factors in arterial hypertension. When treating hypertensive patients many physicians employ measures to reduce the body's content of sodium chloride with the feeling that this will be of benefit whether it is induced by dietary salt restriction, natruretic drugs, or oral cation exchange resins. Genest *et al.*(1) produced evidence to suggest that aldosterone production may be increased in essential hypertension. This could be a part of the mechanism whereby hypertensives retain sodium. Progesterone has been shown by Landau *et al.*(2) to possess not only natruretic properties in normal persons but to oppose the salt retaining influence of aldosterone *in vivo*(3). It appeared worthwhile to assess the influence of progesterone on high blood pressure in experimental animals and in humans with essential hypertension.

Methods and materials. Rats. Growing male rats of the Long-Evans strain were subjected to left nephrectomy and loose ligature of the right renal artery according to the method of Kempf and Page(4). Rat systolic blood pressures were determined by the method of Friedman and Freed(5). Pressures were recorded daily at 8 a. m. before injection of progesterone or placebo and at 4, 6, and 8 hours after injection. Of 2 male mongrel dogs used, one had been rendered hypertensive by wrapping its kidneys in silk to induce perinephritis after the method of Page(6). The other had silica injected into the renal arteries after the method of Moses(7). Blood pressures were determined daily by direct needle puncture of femoral artery and recorded by mercury manometer. Both dogs served as their own controls having been observed to maintain a reasonable elevation of blood pressure for some months before progesterone was administered. Human subjects with a diagnosis of primary arterial hypertension were admitted to the metabolic ward of Lilly research unit in Marion County General Hospital. All patients had been attending the hospital out-patient clinic for several years during which time all known causes for secondary hypertension had been thoroughly excluded. No patient had blood urea above normal. Each patient was given a 1½ g sodium diet, and total intakes of sodium and potassium were calculated daily. Twice daily injections, initially of placebo, later of progesterone, and finally of placebo were given into alternate buttocks throughout hospitalization. Substitutions of placebo for progesterone and vice versa were made without the patients' knowledge. These patients were allowed up but were made to rest in bed for 15 minutes before each blood pressure determination. Blood pressures in lying and standing positions were determined 6 times daily from 6 a.m. at 3 hourly intervals until 9 p. m. Stools were not analyzed for electrolyte content, but daily 24-hour urine specimens were collected and analyzed for sodium, potassium, chloride, and creatinine. Sodium and potassium were estimated by flame photometry using an internal lithium standard, chloride by the Van Slyke modification of Volhard's method(8), and creatinine by the method of Bosnes and Fanasky(9). Three women whose blood pressure remained within the hypertensive range after several days of dietary and placebo treatment were retained in the hospital for trial of progesterone. One man was retained in the series even though his blood pressure fell almost to normal with only dietary and placebo treatment. It was thought worthwhile to study the effects of progesterone on at least the electrolyte metabolism of a hypertensive human male. In all species, progesterone used was Injection progesterone U.S.P., 50 mg/cc.* Each cc contains progesterone 50 mg in peanut oil, with benzyl benzoate 20% and preservative phenol 0.5%. The placebo

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* Supplied by Eli Lilly and Co.

TABLE I. Observations on Patients before, during, and after Administration of Progesterone.

Patient	Before treatment with progesterone			During treatment with progesterone				After treatment with progesterone		
	Days of placebo treatment	Blood pressure		Days of progesterone treatment	Daily dosage of progesterone	Blood pressure		Days of placebo treatment	Blood pressure	
		Lying	Standing			Lying	Standing		Lying	Standing
G.M.	5	187/116	187/125	15	300	152/100	136/97	6	187/113	168/121
N.H.	5	176/117	153/113	14	150	141/102	115/89	17	158/105	130/100
E.C.	6	185/107	173/106	14	300	150/ 96	138/93	16	159/112	152/ 98

* All blood pressures (arterial, in mm Hg) are avg of 12 determinations during last 2 days of indicated periods. Durations of these treatment periods varied as indicated, no changes in treatment being made until B.P. had remained reasonably constant for 2 days.

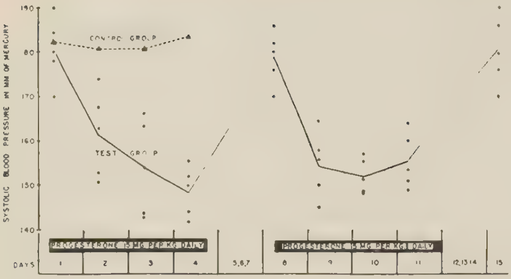
material* differed from this only in that it contained no progesterone.

Results. Rats. Five of 6 hypertensive rats, given progesterone 15 mg/kg subcutaneously once a day for 4 days, exhibited reduction in systolic blood pressure from a high pretreatment level (avg. 181 mm Hg for 5 rats) down to much lower levels (avg. 148 mm Hg for 5 rats). Many individual readings were below this figure in the region of 140 mm Hg. Reduction of blood pressure was not noticeable until second day of progesterone treatment (Fig. 1), and became maximal by the end of third day. The sixth rat failed to respond. Its blood pressure had been within 200 to 220 mm Hg range for some months and had previously responded only minimally to several other drugs. The data pertaining to this animal were not used in Fig. 1. Without exception blood pressures of the 5 responsive rats returned to high pretreatment levels within 2 days after discontinuing progesterone. Three more hypertensive rats were used as controls and received 4 daily injections of placebo. The average of their blood pressures remained at all times within the range of 180 to 185 mm Hg (Fig. 1). These rats were later responsive to progesterone.

After a 3-day interval, progesterone injections were repeated during another 4 days (Fig. 1). The resulting fall in blood pressure was slightly less but of the same order as before (Fig. 1).

To test whether rats might become refractory to the hypotensive influence of progesterone, a long term experiment was also undertaken. Three rats were injected daily for 4 weeks with 15 mg/kg of progesterone. With exception of the first 2 days, their blood pressures were reduced to 150 mm Hg range during the entire 4 weeks. Although their blood pressures fluctuated from day to day and from week to week, no trend towards refractoriness was seen; and the lowest average pressure for any one week occurred during the last week. All blood pressures promptly returned to the high pretreatment levels within 2 days of stopping treatment.

Dogs. Both hypertensive dogs showed considerable reduction in blood pressure at time of progesterone administration (20 mg/kg



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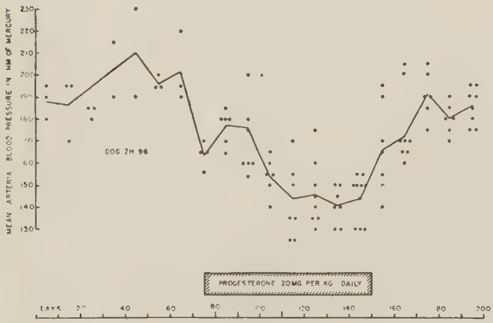

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FIG. 1. The effect of progesterone on the blood pressure of hypertensive rats. Each triangle represents daily avg of 4 blood pressures (B.P.) in all 3 rats treated with placebo injections. Each dot represents daily avg of 4 B.P.'s in individual rats of the progesterone treated group of 5 animals. Lines are drawn through daily avg B.P. for all rats in a group.

FIG. 2. The effect of progesterone on the blood pressure of a hypertensive dog. Each dot represents a single blood pressure (B.P.) determination in dog ZH 96. The line passes through the avg B.P. of 10-day periods.

daily), which was maintained until treatment was stopped (Fig. 2). Changes in dog blood pressures occurred only slowly during the course of weeks, in contrast to the rapid changes which occurred in rats. Fig. 2 relates to one of the dogs and is typical of the response in both animals.

Humans. A reduction in B.P. occurred in all 3 women at time of progesterone treatment (Table I). Without exception, their pressures increased again after reinstitution of placebo treatment. Fig. 3 relates to one of these patients, and her response was typical of all 3 women. Blood pressures reached their lowest level only after 10 to 12 days of progesterone treatment, and this might appear to have followed natruresis and the establishment of a new sodium equilibrium. That standing blood pressures were reduced more than lying blood pressures is also suggestive indirect evidence that the fall was secondary to natruresis.

After placebo was substituted for progesterone, every patient retained sodium in amount about equal to that lost at time of progesterone treatment. Blood pressures did not rise again until this retention was almost complete. In patient G. M., a second series of progesterone injections was given following a cycle such as illustrated by Fig. 3. Only 150 mg of progesterone once daily was given during this second period, and this resulted in less salt loss and a smaller and slower fall in blood pressure than had followed the higher dosage. Refractoriness, shown by sodium retention and rising bood pressure, developed after 14 days. The situation was brought under control promptly after progesterone dosage was doubled to 150 mg twice daily.

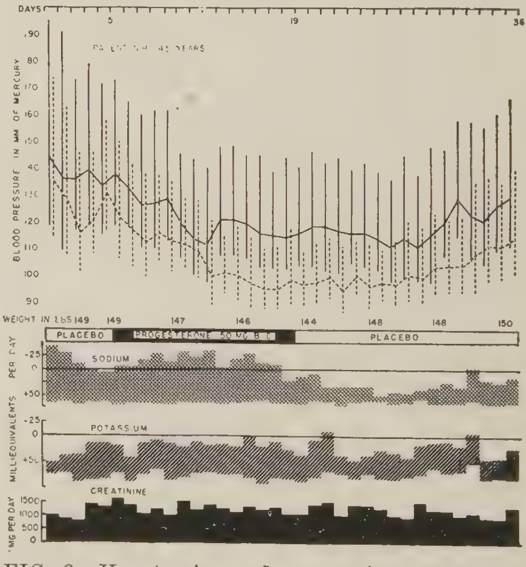

FIG. 3. Hypotensive and natruretic responses to progesterone of a woman suffering from essential hypertension. Tops of vertical bars represent avg of 6 systolic blood pressures (B.P.) in one day while bottoms represent avg of the 6 diastolic B.P.'s. Black bars refer to lying B.P. and interrupted bars to standing B.P. Mean B.P. (diastolic pressure plus ⅓ of pulse pressure) from day to day indicated by lateral lines. Milliequivalents of sodium and potassium passed in 24-hr urines are related to calculated dietary intake. Dietary intake indicated by distance of base of column below zero line. Sodium and potassium columns not reaching zero line represent negative balance.

Immediate natruresis and a slow continuous reduction of blood pressure resulted.

The only man in this series became virtually normotensive during initial control period of placebo treatment. However, he did exhibit a convincing natruresis in response to progesterone which, as in the case of the women, was abruptly followed by sodium retention the day after placebo was substituted for progesterone. His blood pressure changes during progesterone treatment, although necessarily too small to be of significance, were at least in the right direction to be consistent with the response observed in the women.

Discussion. Administration of progesterone to both rats and dogs with experimental hypertension and to human subjects with primary arterial hypertension coincided with a considerable lowering of blood pressure.

The hypotensive action of progesterone in humans might well have been secondary to natruresis. No electrolyte studies were carried out in the animals to study this point.

No refractoriness to progesterone was encountered when dosage was 150 mg twice daily, but it was evident in one patient (G. M.) after 14 days of progesterone at half this dosage.

Even when the daily average of all 6 blood pressures was almost within the normotensive range, single very high pressures in response to emotion were occasionally observed. Such an observation is consistent with there being more than one factor involved in the mechanism of hypertension.

A disadvantage in the use of progesterone was pain at injection sites. This pain was just as evident following placebo and presumably depended upon the bulky nature of the injections. Another disadvantage was the expected fourth day "withdrawal bleeding" experienced by the women after progesterone was discontinued. One woman had her normal menses, no heavier than usual, actually during progesterone treatment. Withdrawal bleedings were no longer nor more copious than the usual menses of these women. Impotence in men following progesterone administration has been reported(10), but the only man in this series had been impotent for some years and experienced no change in this respect after receiving progesterone.

No body depletion of potassium occurred with progesterone induced natruresis, and potassium was even to some small extent retained at this time. In this respect, progesterone appears to have an advantage over naturetic and kaluretic agents such as mercurial diuretics and chlorothiazide.

In no animal or human were blood pressures reduced by progesterone to below normal levels.

Even with these advantages, however, it is not implied that progesterone should be generally used in treating human arterial hypertension. Withdrawal bleeding in women, impotence in men, and painful bulky injections make it impractical.

Summary. (1) Progesterone administration to rats and dogs with experimental hypertension and to humans with primary arterial hypertension resulted in a decline in blood pressure levels. Blood pressures increased once more in all cases after progesterone was discontinued. (2) In humans, but not necessarily in rats and dogs, blood pressure reduction would appear to have resulted from natruresis.

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Received August 31, 1959. P.S.E.B.M., 1959, v102.

Detection of Right-to-Left Circulatory Shunts: A New Method Utilizing Injections of Krypton^{85}. (25283)

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Experimental and clinical experience with krypton^{85}, an inert radioactive gas, has demonstrated that determination of blood levels following its inhalation provides a rapid and sensitive method for detection, localization and quantitation of left-to-right circulatory shunts(1,2). During these studies it was found that when solutions of Kr^{85} in normal saline were injected intravenously, virtually none of the gas could be detected in arterial blood since it had been largely eliminated in its first passage through the pulmonary circulation. These observations suggested that if a high arterial Kr^{85} level was observed following intravenous injection it would be attributable to a right-to-left shunt permitting passage of venous blood into the pulmonary veins, chambers of the left heart or the aorta.

Materials and methods. Eleven mongrel dogs weighing 10-18 kg were employed. Right-to-left shunts were created by anastomosis of left atrial appendage to the pulmonary artery. Anesthesia was induced with intravenous thiopental and following tracheal intubation respiration was maintained with 100% oxygen supplied through a demand respirator. The ventilatory rate was adjusted to 16-24 cycles/minute. The left pleural cavity was entered through the fourth intercostal space, the pericardium widely incised and partially occluding clamps were applied to wall of pulmonary artery and the tip of left atrial appendage. An ellipse of tissue measuring approximately 10 × 3 mm was excised from the excluded segment of pulmonary artery and the tip of the appendage amputated. The anastomosis was then constructed with a continuous over-and-over suture of fine silk. Five animals were studied immediately and 6 at intervals of 6 to 10 days after operation. Upon completion of experiments the atrial appendage was transected in each animal and the patency of the anastomosis determined by inspection and by observation of presence or absence of bleeding from pulmonary artery before death. In 4 dogs the shunt was completely occluded by thrombus and the data obtained in them was grouped with that of control animals. The solution of Kr^{85} was prepared by injecting 15 ml of air containing 3.1 mc of the isotope into a 30 ml vial containing 15 ml of sterile saline solution. Since the solubility coefficient of the gas is 0.045(3) each ml of saline solution initially contained approximately 10 μc of Kr^{85}. Thirty μc of the gas (3-5 ml of solution) were withdrawn for each injection and the volume of saline replaced. In each animal a Cournand catheter was inserted through jugular vein and positioned to allow injections of Kr^{85} into the pulmonary artery, right ventricle, right atrium or superior vena cava. Ten ml blood samples were drawn into heparinized syringes from the femoral artery at various time intervals after injection. The level of radioactivity in arterial blood was determined by counting whole blood sample for one minute in a continuous gas flow Geiger-Muller tube of 10 ml capacity.* The tube was rinsed with saline and compressed air after each determination. Blank samples were drawn immediately prior to each injection. In those animals studied acutely, injections were made both with the shunt open and closed so that each animal could serve as its own control. In 3 dogs without shunts, studies were made in presence of various ventilatory states. Kr^{85} was injected intravenously during slow spontaneous respiration, during complete apnea and immediately after the left lung had been rendered completely atelectatic by compressing it and occluding the main bronchus. In one animal radioactivity of expired air as well as arterial blood was determined with the right-to-left shunt open and closed. The expired air was counted at 10 second intervals by a G-M

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* Model M-4, Nucleonic Corp. of America.

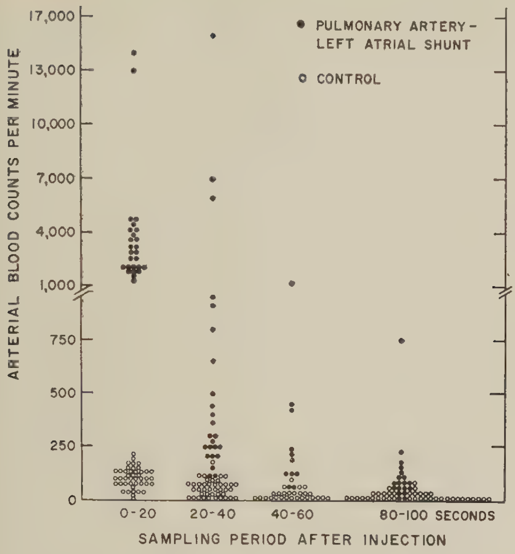

FIG. 1. Kr^{85} levels in arterial blood following intravenous or right heart inj. in dogs with and without right-to-left shunts.

tube† inserted into the endotracheal tube. Serial arterial blood samples were drawn at equivalent intervals.

Results. Background or blank counts did not exceed 50 cts./min. and these values were always subtracted from total counts. Following intravenous, right heart or pulmonary artery injection in animals without right-to-left shunts (normal or with an occluded anastomosis) the radioactivity of arterial blood never exceeded 206 cts/min regardless of sampling period (Fig. 1). With a patent right-to-left shunt counts of arterial blood drawn during the first 20 sec. after injection ranged from 1451 to 14,100/minute. The 2 highest values resulted when flow through the anastomosis was increased by temporary constriction of pulmonary artery distal to it. Arterial samples drawn at later intervals after injection contained less radioactivity than those obtained during the first 20 sec. and the difference between levels in normal and shunt dogs progressively decreased with time (Fig. 1). The advantage of the 0-20 sec. sampling period in detecting a right-to-left shunt is further demonstrated by curves relating arterial radioactivity to time (Figs. 2A and 2B). In this animal a count of 3900 was obtained at 15 sec. when the shunt was open while at this same period after injection, the count was only 340 with the shunt closed. The latter value is somewhat higher than those observed in other control dogs since it represents an integrated 10-20 sec. value while an integrated 0-20 sec. sample was ordinarily drawn (Fig. 1). Both curves illustrate the rapidity with which the gas is cleared from the circulation when ventilation is normal; in the presence of a right-to-left shunt, however, the rate of disappearance is slower and a significant level of Kr^{85} is present in arterial blood as long as 3 minutes after injection. This persistence of the gas is attributable to recirculation through the shunt; only a portion of the isotope is removed by the lungs during each circulation.

The levels of Kr^{85} in expired air are also shown in Figs. 2A and 2B. In the absence of a right-to-left shunt (Fig. 2B) a large amount of gas appeared immediately and its concentration rapidly fell so that background level was reached within 45 sec. When a shunt is present a far smaller quantity of gas appears initially and exhalation of significant quantities is continued for nearly 3 minutes. These differences are also attributable to recirculation of krypton-rich venous blood through the shunt, its continued passage through the systemic circulation and gradual elimination by the lungs.

With slow spontaneous respiration instead of hyperventilation induced by the demand respirator, arterial counts in normal range were observed when no shunt was present. During complete apnea, induced with curare, an average count of 698/minute was obtained in the 0-20 sec. sample indicating that even without air exchange the major portion of gas was cleared by diffusion. With unilateral total atelectasis and bronchial occlusion an arterial level of 2666 c.p.m. occurred in the 0-20 sec. arterial sample indicating that perfusion of the unventilated lung was continuing and resulting in a sizable right-to-left shunt.

Krypton^{85} is readily eliminated from pulmonary arterial blood because of its very low solubility and the high gradient in partial pressure of Kr^{85} existing between blood and alveolar air. This has been confirmed by

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† Model D-34, Nuclear-Chicago Corp.

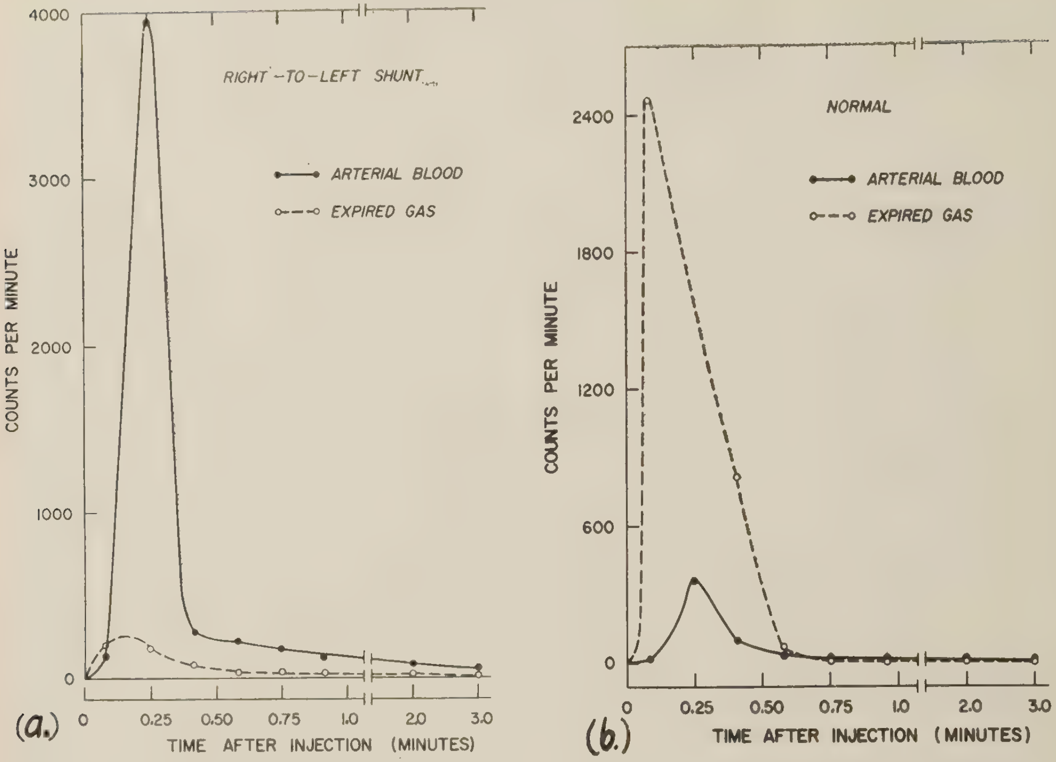

FIG. 2. Concentrations of Kr^{85} in arterial blood and expired air after intravenous or right heart inj. when a right-to-left shunt was functioning (*a*) and occluded (*b*).

recent studies of Chidsey and associates(4).

It is apparent that, in presence of a right-to-left shunt, regardless of its site, high levels of Kr^{35} are present in arterial blood after intravenous injection. This technic may be used to localize the site of origin of the shunt by selectively injecting the isotope into the chambers of the right heart and the pulmonary artery. Injections at or proximal to the shunt may be expected to give high arterial levels while those made distal to it should result in low arterial counts. Data obtained during catheterization in patients with congenital heart disease indicate that the method is useful in this respect.

Summary. Right-to-left circulatory shunts were produced in 11 mongrel dogs by anastomosis of the left atrial appendage to the pulmonary artery. When injections of Kr^{85} in solution were made proximal to the shunt the level of radioactivity in arterial blood drawn during the first 20 sec. after injection was always greater than 1400 cts./min. In dogs without shunts, however, similar injections resulted in arterial blood counts of 206/min. or less. The technic thus provides a new method for detection and localization of veno-arterial shunts.

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Received September 8, 1959. P.S.E.B.M., 1959, v102.

Prolongation of Skin Homograft Survival in Rabbits by 6-Mercaptopurine.*† (25284)

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Schwartz and his associates, have recently reported that intramuscular injection of 6-Mercaptopurine in doses of 3 mg/kg and 6 mg/kg produces a profound suppression of humoral antibody formation to crystalline bovine serum albumin in rabbits when the antigen and antimetabolite are given simultaneously(1). There was no effect on the secondary immune response to bovine serum albumin when a dose of 6 mg/kg of 6-Mercaptopurine was employed(2). Since other physical and chemical agents such as x-irradiation and cortisone(3,4) which suppress formation of antibody also modify the homograft response it was felt profitable to examine the effect of 6-Mercaptopurine on survival time of skin homografts. This paper presents data which indicate that 6-Mercaptopurine can modify the homograft response to skin homotransplants in rabbits although it does not completely abolish it.

Method. In 3 experiments reciprocal cross-grafts were exchanged between albino New Zealand strain rabbits (weighing 2.5-3.0 kg) and black-and-white Dutch strain rabbits (weighing 1.0-2.2 kg) randomly distributed as to sex, which were obtained from commercial sources and maintained on a stock *ad lib.* diet. Both strains were equally distributed among treated and control groups. Animals were anesthetized with I.V. nembutal® and 12 cm² free full thickness skin grafts were removed from the upper and lower abdomen and the graft site prepared with alcohol. Homografts were placed in the upper site and autografts in the lower site. Grafts were sutured in place with 4 interrupted 6-0 silk sutures at the corners and a continuous suture around the margin. A thin layer of bacitracin® ointment was applied to the wound edges after which the grafts were covered with gauze squares held in place by several turns of quick drying plaster of Paris bandage. Grafts were inspected daily and the gross condition noted. The endpoint of graft rejection was invariably abrupt and clearcut. 6-Mercaptopurine (6-MP) was administered intravenously *via* the ear veins daily from a freshly prepared stock solution containing 15.2 mg/cc. The following dosage schedules were employed. Group 1 animals received a daily dose of 6 mg/kg. 6-MP starting on the day of grafting and continuing until the graft sloughed. In this group pretreatment and weekly white blood cell counts were done on all surviving treated animals. Several homografts exhibiting long term survival were examined under the dissecting microscope for capillary blood flow according to the method of Taylor and Lehrfeld(5). Microscopic sections of homografts stained with hematoxylin and eosin and methyl green pyronin stains were examined for inflammatory infiltrate suggestive of rejection when an animal died with a homograft in good condition. All animals were weighed at 4-5 day intervals. Group 2 animals were treated with 12 mg/kg of 6-MP daily starting on the day of grafting and continuing until slough of the graft occurred. Pre-treatment and weekly white blood cell counts were done on all treated animals and they were weighed at 4-5 day intervals. Group 3 animals were treated with 12 mg/kg 6-MP starting on day of grafting and continuing for a period of 14 days. In this group graft survival was the only observation made.

Results. The data are summarized in the chart. At a dose level of 6 mg/kg employed in Group I rabbits, there was a barely discernible effect of 6-MP on any prolongation of viability in the graft. Serial observations made

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* Aided by grants from U.S.P.H.S., Minn. Heart Assn., Am. Heart Assn. and Minn. Division of Am. Cancer Soc.

† Supplied through courtesy of Donald S. Searle, Burroughs, Wellcome and Co.

TABLE I. Effect of 6-MP on Survival of Rabbit Skin Homografts.

Group	Effect on body wt	Effect on peripheral blood counts	Mortality	Effect on homograft survival
1 6 mg/kg/day from day of grafting until slough	No significant loss of wt in either treated or non-treated rabbits	Slight fall in Hgb and WBC after 2 wk treatment	4 deaths in 10 treated animals; no deaths in controls	Mean slough time in treated animals of 17.5 days (15-25) for 6 animals; mean slough time of 14.4 days (11-16) for 10 untreated animals. 4 animals died at 5, 12, 15, and 27 days with viable grafts.
2 12 mg/kg/day from day of grafting until slough	Progressive wt loss in all but 1 treated animal	Fall in circulating WBC and Hgb max (WBC=400 –6650) in animals surviving 3 wk or longer	7 deaths in 10 treated animals; no deaths in controls	Mean slough time of 22 days (19-25) in 3 treated animals; mean slough time of 14.1 days (13-17) in 9 controls. Seven animals died with viable grafts at avg of 18.4 days (10-28).
3 12 mg/kg/day from day of grafting to 14th day post-grafting	Not recorded	Not recorded	1 death in 10 treated animals; no deaths in controls	Mean slough time in 9 treated rabbits, 24.3 days (23-26); mean slough time of 9 untreated rabbits, 14.1 days (13-17). One treated animal died at 23 days with a viable graft.

at daily intervals of the gross condition of homografts in treated and untreated rabbits disclosed no significant differences in a majority of the animals. However, two treated animals showed prolongations of graft survival to about twice the length of survival time of untreated animals. Mortality due to treatment was not unduly high in this group. Moreover, profound leukopenia was not attained in any of the animals so treated. Histologic sections of homografts showing long term survival revealed extensive round cell infiltration. This contrasts with the absence of significant inflammatory infiltrate in autografts from the same animals. In the absence of gross infection we interpret this as evidence for an active process of graft rejection in these animals. In the next group a dose level of 12 mg/kg was employed. It is readily apparent from the summary of the results that the mortality due to treatment was greatly increased. This prevented the collection of data on graft survival since all animals who died had viable grafts. Nonetheless, 4 out of 10 treated animals had prolongations of graft survival to almost twice that of untreated animals and all died with viable grafts. Capillary blood flow was observed in the graft of the longest surviving animal at 27 days. Examination of histological sections of homografts and autografts from those animals succumbing with viable grafts failed to disclose any outstanding differences. Grossly the grafts had regrowth of hair at time of death. In this group and a subsequent group it was apparent from gross observation that the condition of the grafts in treated animals was better than grafts in untreated animals at comparable times after grafting. All of these features indicate a suppression of the host response to homologous skin.

The dose schedule of Group III was devised with the idea of reducing the mortality due to treatment by stopping treatment at 14 days. It had been observed previously that most animals died after the second week of treatment. A clear-cut demonstration of retardation of the homograft response was achieved. An interval of more than a week supervened after the slough of the last homograft in the untreated group and the slough of the first homograft in the treated group. Mortality due to treatment was significantly reduced. Nonetheless, all homografts were rejected between the 26th and 28th day following grafting. Hence, it is apparent that additional treatment was required in order to maintain suppression of the homograft rejection response.

Discussion. These results clearly established that 6-Mercaptopurine in the dose levels employed produced an inhibitory effect on the immune response to skin homografts in rabbits. The lethality of this treatment with 6-Mercaptopurine has also been demonstrated. That lethality was not always a result of bone marrow suppression is indicated by the relatively large number of animals dying prior to development of leukopenia. The unusually large number of animals dying prior to slough of the skin homograft did not permit determination of maximum survival time if treatment had been continued indefinitely. If treatment were discontinued, as in Group III, all grafts eventually sloughed. Hence, it appeared that sustained treatment was necessary to maintain suppression of the homograft response. It was also of interest to note that profound leukopenia was present in those animals showing the longest graft survival. This was interpreted as suggesting the importance of using doses sufficiently large to achieve hemopoietic suppression in order to obtain this effect with this antagonist of purine metabolism. Nonetheless, interference with nucleic acid synthesis(6) may be inferred to be the predominant action of this drug and the means by which it presumably suppresses production of humoral antibody and the homograft reaction.

Summary. 6-MP, a powerful metabolic antagonist to nucleic acid synthesis, prolongs survival time of skin homografts in rabbits when administered after grafting. Treatment must be given continuously to maintain graft viability. However, toxicity of 6-MP limits the length of time treatment may be given.

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Received September 10, 1959. P.S.E.B.M., 1959, v102.

Formation of Mixed Crystals of Cholesterol and Sitosterol *in vitro* and in Rabbit Intestine. (25285)

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Many investigators have observed that simultaneous feeding of plant sterols and cholesterol to animals prevented hypercholesteremia obtained when only cholesterol was fed. These researches demonstrated the effectiveness of sitosterol as a hypo-cholesteremic agent in man. The following modes of action have been proposed for hypo-cholesteremic effect of sitosterol: (1) Formation of a mixed crystal of sitosterol and cholesterol, from which cholesterol is less readily dispersed into the colloidal form(1,2); (2) competition between sitosterol and cholesterol for esterification(3); and (3) competition with cholesterol for acceptor sites on or within the intestinal mucosal cell(4,5). Dispersion into colloidal

TABLE I. *In Vitro* Conversion of Mixed Aqueous Suspensions to Mixed Crystals.

Composition of equilibration mixture	Time of equilibration (hr)	Identity of precipitate by x-ray diffraction
.25 mg/ml sitosterol (parent suspension)	9	Sitosterol + mixed crystal (weak)
.25 mg/ml cholesterol (parent suspension)	20	As above
5 mg/ml sodium desoxycholate		
.1 M phosphate buffer (pH 8.8)		
.20 mg/ml sitosterol (representing parent suspension with largest 20% of particles removed)	9	Sitosterol (weak) + mixed crystals (weak)
.20 mg/ml cholesterol (parent suspension)	20	Sitosterol (weak) + mixed crystal
5 mg/ml sodium desoxycholate		
.1 M phosphate buffer (pH 8.8)		
.12 mg/ml sitosterol (representing parent suspension with largest 52% of particles removed)	9	Mixed crystal (weak)
.12 mg/ml cholesterol (parent suspension)	20	Mixed crystal (strong)
2.5 mg/ml sodium desoxycholate		
.1 M phosphate buffer (pH 8.8)		

form and esterification are presumed to be requirements for sterol absorption. In our experiments it was possible to demonstrate in a model colloidal aqueous system and in intestines of rabbits conversion of mixtures of sitosterol and cholesterol to the characteristic 1:1 mixed crystals previously described(2).

Methods. In vitro experiments: Aqueous sterol suspensions were prepared as previously described(2). Sitosterol suspensions were much less stable than cholesterol suspensions of the same concentration. In our experiments particle size of sitosterol was a consideration since rapid equilibration was desired. Consequently, the largest particles were removed by degrees from initial sitosterol suspension by centrifugation. After gravimetric analysis of the supernatant fine suspension, the stated mixed suspensions (Table I) were placed in rubber-stoppered test tubes and gently rocked in a bath at 38°C. Samples for x-ray diffraction were obtained by centrifuging at 60,000 × G for 30 minutes, washing with water to remove soluble materials, and re-centrifuging. Diffraction patterns were compared with those of pure compounds and of an equimolar composition crystallized from the molten state or from methanolic solution. *In vivo* experiments: To minimize interference in x-ray diffraction patterns by insoluble solids present in a natural diet, a purified diet described by Thacker(6) was modified by excluding fiber. Two % cholesterol and 8% sitosterol were added to the diet in finely ground form, and mechanically mixed with the diet. Male, adult albino rabbits were allowed access to the diet and water *ad lib* for 2-3 weeks. Animals were then sacrificed by inducing an air embolism. The small intestine was immediately removed and divided into 2 equal segments. The lumen of each segment was stripped of contents and the intestine then slit longitudinally to wash the villi with water. Thus, 2 separate specimens were obtained from each intestinal segment.

Results. With preliminary experiments, the pellet obtained by vigorous centrifugation of intestinal contents or washings from the villi, yielded x-ray diffraction patterns of crystalline sitosterol or no pattern at all. Initial failure to obtain a pattern may be attributed to presence of large amounts of amorphous material, *e.g.*, cellular debris, mucosa, etc. Subsequent experiments in which a preliminary, mild centrifugation was used to remove large particles and in which the pellet used for diffraction analysis was obtained from a vigorous centrifugation of the resulting supernatant were more successful.

A 1:1 cholesterol-sitosterol mixed crystal pattern was obtained from either intestinal contents or villi washings irrespective of the intestinal segment in several of the animals. Mixed crystal patterns ranged from strong to weak and, also, were seen in some specimens in the presence of the sitosterol pattern. A particularly strong mixed-crystal pattern was obtained from both the intestinal contents and

TABLE II. Comparison of X-Ray Diffraction Patterns.

d, A.U.	Sitosterol	Cholesterol	1:1 mixed xtal standard	*In vivo* centrifugate	*In vitro* centrifugate
18.5	*3*— .6				
5.89	*1*—1.0				
5.72		*1*—1.0	*2*— .3	*2*— .3	*2*— .3
5.48			*1*—1.0	*1*—1.0	*1*—1.0
5.41		*4*— .6			
5.33			*3*— .2	*3*— .2	*3*— .2
5.21		*2*— .8			
4.99		*3*— .7			
4.85	*2*— .8				
4.61	*4*— .4				

Table II presents the interplanar spacings and relative intensities for the strongest lines obtained from x-ray diffraction patterns. Italicized numbers refer to intensity rank of that diffraction line while the following number is relative intensity, I/I_1. The uncertainty of d values varies from ±.01 to ±.03 Angstrom unit depending on sharpness of the line.

villi washings of the second segment in one animal. In no case was a cholesterol pattern obtained. A comparison of the x-ray diffraction patterns obtained *in vivo* and *in vitro* is presented in Table II.

Discussion. Davis(2) previously observed that both sitosterol and crystals of 1:1 cholesterol-sitosterol mixed crystal have only one-third the solubility of cholesterol in methanol, and exhibit similarly reduced solubilization in aqueous sodium oleate or sodium desoxycholate systems. Sitosterol thus physically removes cholesterol from the dispersed state necessary for absorption. These experiments present evidence that formation of a mixed crystal of cholesterol and sitosterol may indeed be a mode of action responsible for the hypo-cholesteremic effect in ingested plant sterols. The relative importance of other proposed mechanisms is undergoing further investigation.

Summary. It was shown that a 1:1 cholesterol:sitosterol mixed crystal are formed not only in aqueous *in vitro* systems, but also *in vivo*.

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Received September 14, 1959. P.S.E.B.M., 1959, v102.

Oxidative Pathways in Pancreatic B Cells.*† (25286)

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In previous work(1,2,3) glucose-6-phosphatase was demonstrated within the pancreatic B cells in rabbits, guinea pigs and dogs, as well as in ductular epithelium of the dog. Presence of this enzyme in the B cell as well as known direct relationship between glycemic level and insulin production led to the hypothesis that the net amount of glucose-6-phosphate available for B cell metabolism regulates rate of insulin output. Since glucose-6-phosphate is the first step in entrance of glucose into the metabolic cycle, a histochemical study was undertaken of pathways for glucose oxidation within the B cells. Me-

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* Supported by Grant from Nat. Science Fn., Washington, D.C.

† Microphotographs were prepared by Herbert A. Fischler.

tabolism of glucose-6-phosphate could proceed by either the hexosemonophosphate (HMP) pathway or by the Embden-Meyerhoff (E.M.) pathway and the Krebs cycle. For the HMP pathway the triphosphopyridine nucleotide (TPN) linked enzyme glucose-6-phosphate dehydrogenase (G-6-PD) as well as TPN diaphorase (TPND) were studied. Since E.M. enzymes are mostly diphosphopyridine nucleotide (DPN) linked, lactic dehydrogenase as well as DPN diaphorase (DPND) were studied. Succinic dehydrogenase (SD) was utilized as an indicator for Krebs cycle activity.

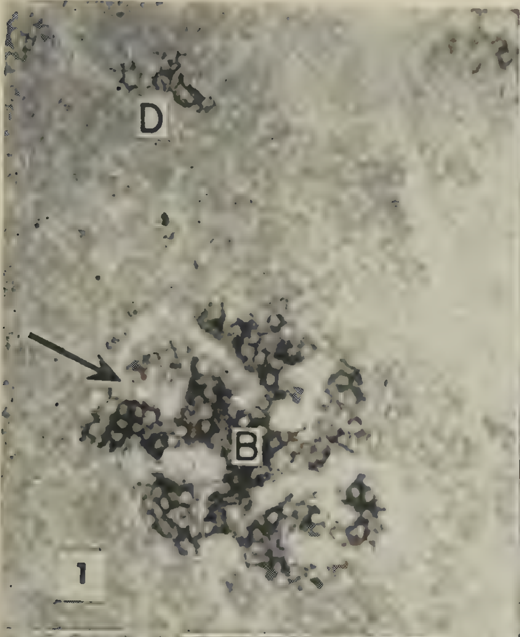
Material and methods. New Zealand white rabbits of either sex weighing 2.5 to 3.5 kg were sacrificed by overdosage with nembutal. The pancreas was removed immediately and frozen in petroleum ether, kept at –70°C by dry ice(4). The frozen tissue was dried with filter paper and stored in stoppered tubes at –20°C. Cryostat sections at 10 μ were affixed to cover slips and incubated immediately (G-6-PD and SD) or placed in neutral 6% calcium formol at –4°C for 15 minutes prior to incubation (TPND, DPND and LD). Additional blocks of pancreas were placed in Zenker formol solution and processed as described previously(5). A modification of the method of Hess *et al.*(6) was used to demonstrate G-6-PD and LD activity. The incubating solution consisted of:

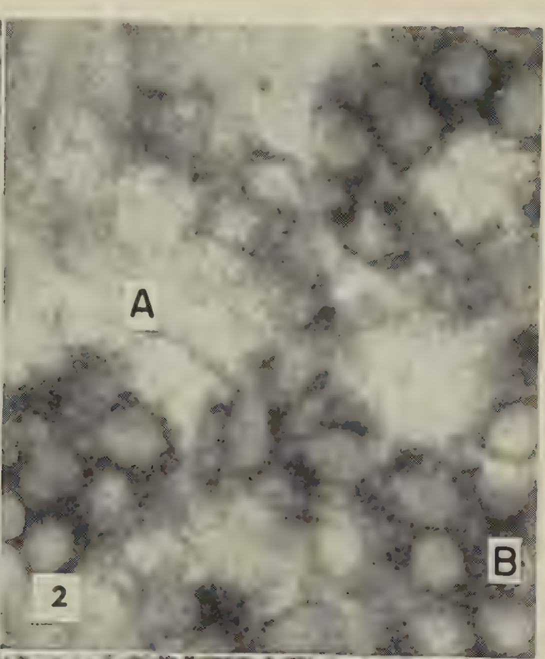
	ml
pH 7.6 phosphate buffer (0.1 M) with sodium cyanide (2 mg %)	1.5
Nitro B-T (3 mg/ml)	1.5
Magnesium chloride (.05 M)	.6
Glucose-6-phosphate potassium salt (.1 M) or sodium lactate (.5 M)	1.2
TPN (25 mg/ml) or DPN (50 mg/ml)	1.2

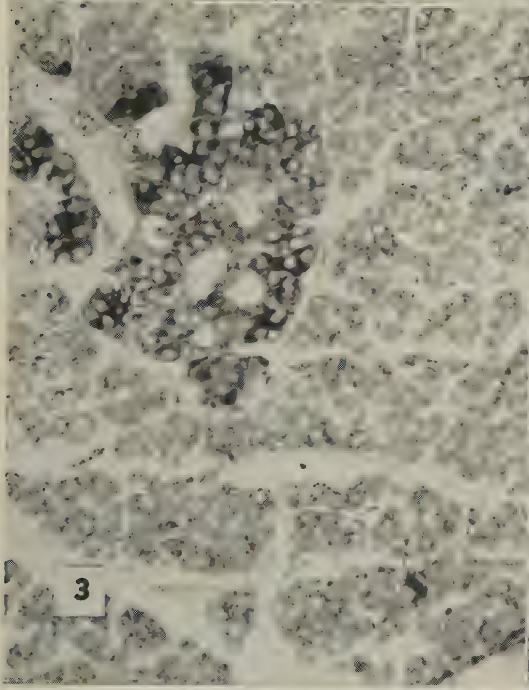
For SD the incubating medium consisted of 2 cc each of the buffer, Nitro B-T solution and 0.5 M sodium succinate. For TPND and DPND a modification of Scarpelli's method (7) was used. The solution consisted of 1.25 cc each of buffer and Nitro B-T, to which is added 2.5 cc of distilled water and 30 mg of either TPNH or DPNH. After addition of the reduced pyridine nucleotide the solution is stable for only 1½ hours. For osmotic protection of tissue, 7.5% polyvinylpyrrolidine was added to each medium(6). For DPNH and TPND sections were incubated 15 to 20 minutes. For dehydrogenases 1 to 1½ hours gave best results. Control sections were incubated in media without substrates. After incubation sections were rinsed briefly in distilled water and fixed in 10% neutral formalin overnight. They were then washed in running tap water for 30 minutes and rinsed in distilled water. Alternate sections were mounted in glycerine jelly and the remainder dehydrated in cellosolve, cleared in toluene and mounted in permount.

Results. G-6-PD staining was most marked in B cells of the islets and in the ductular epithelium (Fig. 1). Abundant, small granules could be seen throughout the cytoplasm of these cells (Fig. 2). When slides are mounted in aqueous media the A cells are identifiable, stained pale brownish red by monoformazan and showing refractile globules packed in the cytoplasm. G-6-PD activity is seen as a few sparse coarse blue granules. D cells could not be identified. The reaction in the acinar tissue was similar to that in A cells. The wall of arterioles showed numerous small granules apparently in the cytoplasm of smooth muscle cells.

Distribution of TPND activity differed from that of G-6-PD in that all pancreatic elements showed greater reactivity. The cytoplasmic structures were morphologically similar to those showing G-6-PD activity but in A cells and in acinar cells they were more abundant and of a darker color (compare Figs. 1 and 3). With some exceptions, LD showed distribution and localization of staining similar to DPND (Figs. 4, 5). However, in each instance, DPND activity was greater than that of LD. Both showed marked staining of densely packed, fine cytoplasmic granules in duct epithelium and somewhat less intense staining in the form of fine granules in acinar cells. The islets showed minimal reactivity except for well stained groups of A cells (Fig. 6). Arteriolar walls had numerous reactive granules which were more numerous after DPND staining than after LD staining. In addition, after staining for DPND, acinar cell walls could be seen as blue outlines.







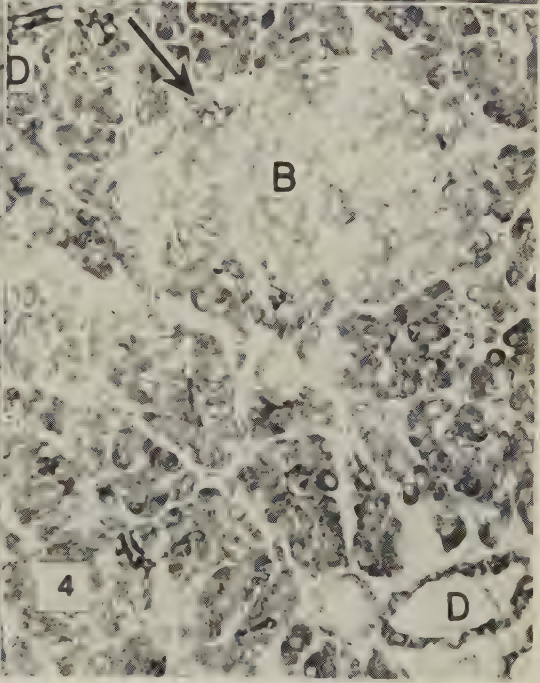
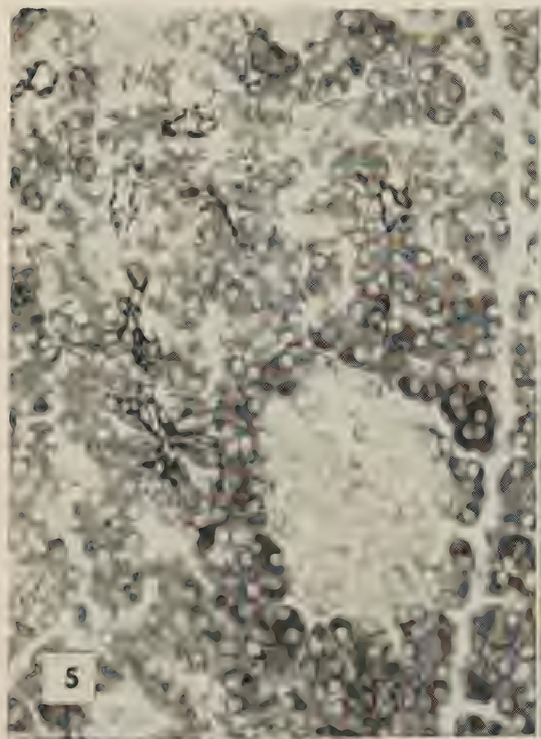


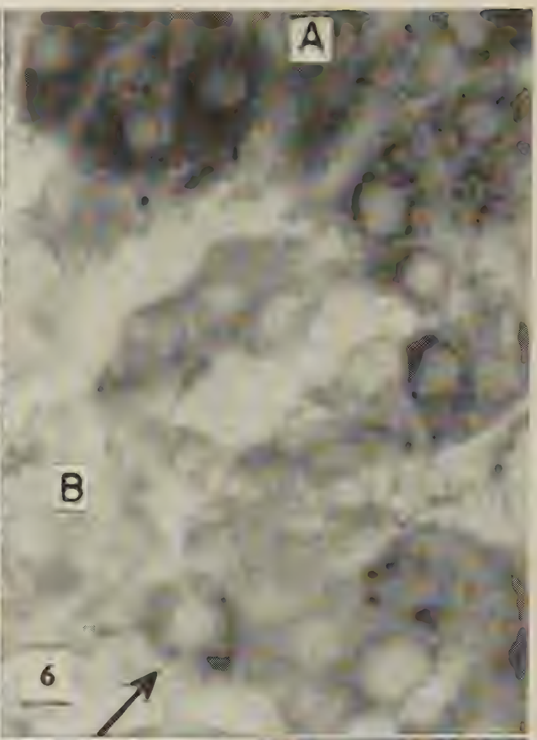
FIG. 1. Rabbit pancreas showing distribution of glucose-6-phosphate dehydrogenase. There is marked activity in B cells (B) as well as in duct epithelium (D). Areas of islets occupied by A cells (arrow) as well as the acinar tissue show limited staining. × 100.

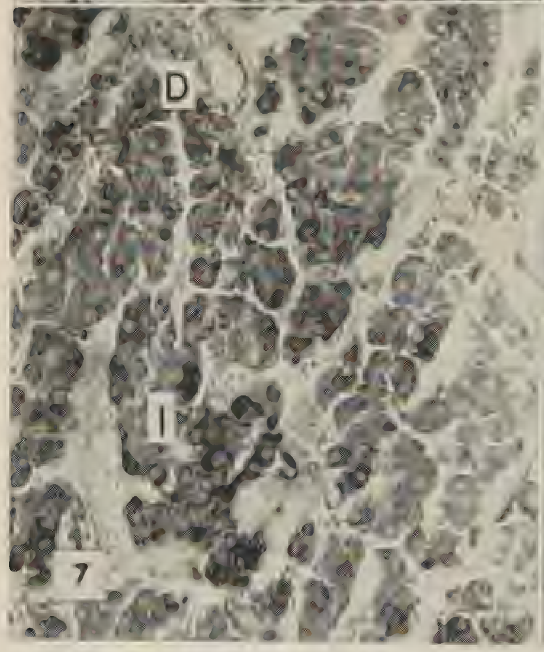
FIG. 2. High power view of same pancreas as in Fig. 1 showing densely packed cytoplasmic granules in B cells (B) and sparsely staining granules in A cells (A). × 960.

FIG. 3. Rabbit pancreas showing distribution of triphosphopyridine nucleotide diaphorase. Note similarity of localization to that of glucose-6-phosphate dehydrogenase. × 150.

FIG. 4. Rabbit pancreas showing distribution of lactic dehydrogenase. Most marked activity is in duct epithelium (D), acinar tissue and A cells (arrow). B cells (B) stain very lightly. × 150.







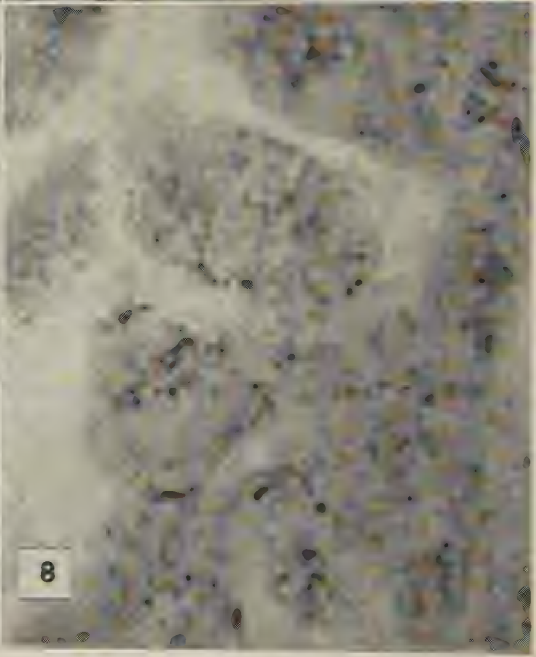


FIG. 5. Rabbit pancreas showing distribution of diphosphopyridine nucleotide diaphorase. Note similarity of localization to that of lactic dehydrogenase. × 150.

FIG. 6. High power view of same pancreas as in Fig. 5 showing faintly staining B cells (B), heavily stained A cells (arrow) and well stained acinar tissue (A) . × 960.

FIG. 7. Rabbit pancreas showing distribution of succinic dehydrogenase. There is good activity throughout but most marked in duct (D) and islets (I). × 150.

FIG. 8. High power view of same pancreas as in Fig. 7 showing mitochondria-like appearance of the reactive structures. × 1280.

S.D. activity was distributed throughout the pancreas. It was most intense in duct epithelium and in B cells, less so in other cells (Fig. 7). The walls of arterioles contained numerous rather coarse granules. The intracellular localization of S.D. differed from that of other enzymes studied. The reactive structures appeared as varying sized rods as well as spheres suggestive of mitochondria (Fig. 8).

Discussion. Our results indicate that G-6-P oxidation in rabbit pancreatic B cells is predominantly *via* the HMP shunt while the EM pathway plays a secondary role. The well marked S.D. activity found in the islets is at variance with results of biochemical studies in toadfish(8). The low ratio of S.D. to cytochrome oxidase is probably accounted for by the limited metabolic role which the EM pathway and the Krebs cycle plays in B cells as compared with the HMP shunt.

The high level of all enzyme activity in duct epithelium is surprising in view of their lack of secretory activity. However, it would be in accord with the fact that these are comparatively primitive cells, as demonstrated by neogenesis of islet cells and acinar tissue from ducts in both the adult animal and the embryo(9,10,11). The relatively greater activity and wider distribution of TPND than of G-6-PD denotes the presence of additional TPN-linked enzymes both within the G-6-PD containing cells and at other sites(6,12,13,14). The relative activity and distribution of LD and DPND are similarly interpreted.

Both biochemical and histochemical studies (7,15,16,17,18,19) have shown that S.D. activity is limited to mitochondria. Morphology of the reactive structures for this enzyme in the pancreas are in accord with this viewpoint. On the other hand, the intracellular localization of the other 4 enzymes has not been clarified. Some have claimed that these oxidative enzymes also are localized to mitochondria(6,7,18,20). However, human erythrocytes which lack mitochondria metabolize glucose by the HMP pathway(21,22,23,24). Furthermore, in studies on cell homogenates these enzymes have been reported in both mitochondrial and extramitochondrial fractions(25,26). In pancreatic cells they showed up as abundant, generally small, closely packed granules diffusely distributed in the cytoplasm. Furthermore, DPND showed intense staining of acinar cell borders. These findings suggest that they are mostly extramitochondrial.

High HMP activity is found in other cell systems which are actively synthetic, as in liver, lactating mammary gland and adrenal cortex(27,28,29). This pathway may serve, not only to supply energy for metabolic processes but, also, as a source of TPNH. It has been claimed that cell synthetic activity may depend on generation of TPNH(30,31) and a role for it in insulin synthesis may be postulated. It is also feasible that TPNH production controls insulin output by influencing the biological activity of the many enzymes which depend on presence of free sulfhydryl groups, or by maintaining glutathione in the reduced state(27). The possible importance of sulfhydryl groups and of glutathione in B cell metabolism has been stressed in connection with the toxic action of alloxan (8). Furthermore, it has been shown in red blood cells that high levels of G-6-PD are necessary for the stability of reduced glutathione(24).

Summary. 1) A histochemical study has been conducted of the distribution of glucose-6-phosphate dehydrogenase (G-6-PD), triphosphopyridine nucleotide diaphorase (TPND), lactic dehydrogenase (LD), diphosphopyridine nucleotide diaphorase (DPND) and succinic dehydrogenase (SD) in the pancreas of the rabbit. G-6-PD was found principally in B cells and duct epithelium; LD in duct epithelium, acinar tissue and A cells. TPND was present throughout, with greatest activity in B cells and in duct epithelium and good activity in other areas. SD activity was present throughout the pancreas with greatest staining in B cells and duct epithelium. The reactive structures for SD seemed to be mitochondrial while the other enzymes apparently are mostly extramitochondrial. 2) These findings demonstrate that glucose metabolism in B cells is predominantly *via* the hexose monophosphate shunt, and indicates a role for it in insulin synthesis. A relationship between TPNH generation by this pathway and avail-

ability of reduced glutathione or of free SH groups in enzymes is considered as a possible mechanism controlling B cell insulin output.

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Received September 16, 1959. P.S.E.B.M., 1959, v102.

Essential Growth Factor in Serum Dialysate for Chick Skeletal Muscle Fibroblasts.* (25287)

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Growth requirements for mammalian cells in tissue culture have been investigated extensively by Eagle and his associates(3). A number of established cell lines will proliferate indefinitely as monolayers in the presence of 13 essential amino acids, 8 vitamins, 6 ions, and a suitable carbohydrate source, provided that dialyzed serum is added to the culture medium(5,6). Freshly isolated monkey kidney cells have similar short term requirements although indefinite cultivation on Eagle's medium and dialyzed serum has not been achieved(4). Our comparable experiments on chick cells have shown that monolayer cultures obtained from skeletal muscle fail to grow or survive in dialyzed serum supplemented by Eagle's medium. Subsequent work as detailed below indicates that these cells require one or more additional factors present in serum dialysate but not occurring in Eagle's medium or other synthetic nutrients presently available.

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[*] This investigation supported by Cancer Research Funds of Univ. of California and by grant from U.S.P.H.S.

[†] With technical assistance of Irene Cady.

Material and methods. Freshly isolated chick skeletal muscle fibroblasts were used. Procedures for routine preparation and management of these cells have been described previously(9,11). Stock monolayer cultures were maintained in 10% undialyzed chicken serum (Colorado Serum Co., Denver), 50% Medium 199(16), and 40% balanced saline (Earle's solution) containing 0.4 mg/ml spleen ribonucleoprotein fraction(11). Cell suspensions were prepared by trypsinization and used to inoculate experimental series in T-15 flasks or 1-oz. prescription bottles. A minimal medium designated CS 199 (10% dialyzed chicken serum, 50% Medium 199, 40% Earle's solution) was employed for these cultures and variously supplemented as described below. Dialyzed serum was prepared at 4°C in standard cellophane casings, using platform shaker and frequent changes of a low-bicarbonate saline (Gey's solution) over a 3 day period. Experimental cultures were maintained at 38°C with a daily 50% fluid change, and terminal determinations of cell number were made with a Coulter electronic cell counter(1,10).

Results. A well defined deficiency syndrome develops if dialyzed serum is substituted for whole serum in monolayer cultures of chick skeletal muscle fibroblasts, despite the presence of a synthetic nutrient (Medium 199) at 50-90% in the supernatant fluid. Cells placed in dialyzed serum were at first similar in appearance to populations in undialyzed medium, and proliferated normally for a day or two. Growth then tapered off in dialyzed CS 199 and ceased after 3 to 4 days. Cells in these cultures rounded up or assumed abnormal shapes, became granular and necrotic, and failed to proliferate if subcultured in the same medium. These changes are illustrated by Fig. 1, which shows typical appearance of skeletal muscle fibroblasts after one subculture in dialyzed CS 199. Control cultures in undialyzed chicken serum grew actively to form a continuous sheet of broad, flat cells, and could be subcultured without significant change in appearance of these cells, or diminution in growth rate. It should be pointed out that extensive growth in undialyzed CS 199 occurred regularly but was not observed previously using chicken serum from other commercial sources(11). The basis for this difference in sera is under investigation, but preliminary studies show that dialysis of all sera tested blocks growth and prevents maintenance of chick skeletal muscle fibroblasts.

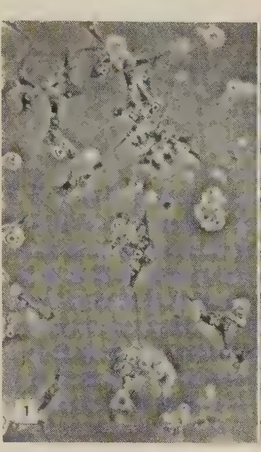

FIG. 1. Typical deficiency state resulting from absence of serum dialysate factor(s). Chick skeletal muscle fibroblasts in 10% dialyzed chicken serum + Medium 199, second passage. Living culture, phase contrast, ×124.

FIG. 2. Prevention of deficiency state by supplementation with proteose-peptone (.1%). Media and cells otherwise similar to culture shown in Fig. 1. Second passage, living culture, phase contrast, ×124.

The deficiency syndrome just described was not corrected by substituting a more complex synthetic nutrient, NCTC 109(13) for Medium 199 in the dialyzed assay medium. On the other hand a deficiency state could be completely prevented if serum dialysate was incorporated at an appropriate level in the initial dialyzed CS 199 Medium. These observations suggest that Medium 199 and NCTC 109 as well as the simpler nutrient of Eagle(3,6) are all deficient in one or more factors occurring in serum dialysate, and which are essential for growth and maintenance of chick skeletal muscle fibroblasts in monolayer cultures.

Of various other supplements tested as sources for the missing serum dialysate factor(s), proteose-peptone (Difco) and to a lesser extent tryptose phosphate broth (Difco) proved positive. Addition of proteose-peptone to dialyzed CS 199 gave a clear-cut effect in terms of cell appearance (Fig. 2) as well as growth stimulation (Fig. 3). At optimal level of 0.1% proteose-peptone the corresponding cell population was indistinguishable in appearance or growth rate from control populations in undialyzed media.

In contrast to the foregoing, negative results were obtained by adding to dialyzed CS 199 supplementary cholesterol, inositol, or vit. B_{12}, all of which have been described as strategic factors for growth of some mammalian strains(6,17,7). Supplementation of dialyzed CS 199 with spleen nucleoprotein fraction(11) also failed to correct the deficiency syndrome. Table I provides a summary of the experiments described above.

Discussion. Failure of 2 synthetic nutrients (Medium 199 and Medium NCTC 109) to correct deficiencies of dialyzed chicken serum indicates that additional requirement of chick skeletal muscle fibroblasts is not to be found in the more extensive arrays of amino acids, vitamins, and other defined factors present in Medium 199 and NCTC 109 as compared to Eagle's medium. It is significant in this connection that Medium 199 alone, or its subsequent modification, M150(14) does not support progressive growth of fresh explants from chick embryonic heart, or even their survival beyond a limited period(15). The effects of proteose-peptone and tryptose phosphate suggest that the missing factors may include peptides or other split products of protein cleavage. Stimulation of chick cells by proteoses and peptic digests is well documented in the older literature on plasma cultures(2,19), although deficiency states were not described. More recently Gerarde and Jones(8) reported that proteose-peptone, but not an amino acid mixture, increased collagen synthesis for cultures of chick lung in dialyzed media. Peptones or proteose-peptone may also stimulate growth of mammalian cells, although not essential for growth of established strains(12,18).

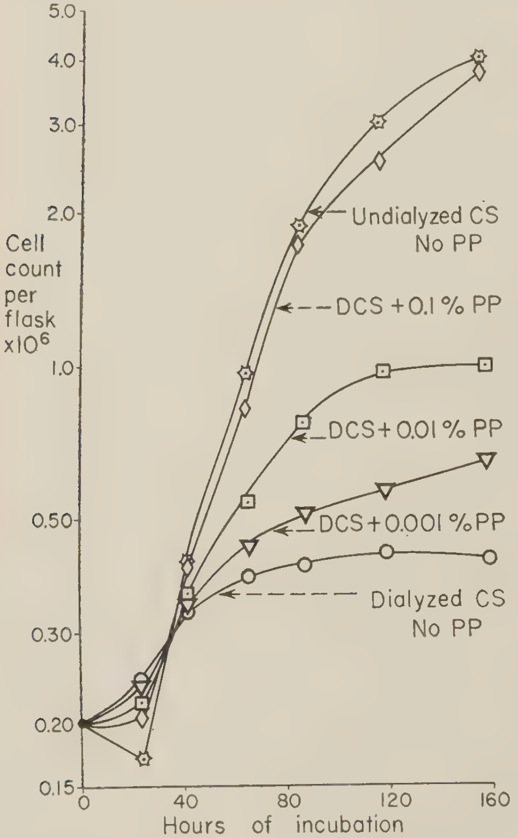

FIG. 3. Growth of skeletal muscle fibroblasts in 10% dialyzed chicken serum (DCS) with 50% Medium 199 and graded levels of proteose-peptone (PP).

TABLE I. Growth of Chick Skeletal Muscle Fibroblasts as Monolayer Cultures in Dialyzed and Undialyzed Media.

Basal medium*	Supplement	Increase in cell count per flask at 5 days, × 10^6†
Undialyzed CS + Medium 199		1.09
Dialyzed CS + Medium 199		.26
Idem	Chicken serum dialysate (1:1), 30% v/v	.97
''	Proteose-peptone, .05%	1.30
”	Tryptose phosphate broth, 10% v/v	1.16
''	Inositol, 10^{-5} M	.30
''	Cholesterol, 1 μg/ml	.29
''	Vit. B_{12}, ”	.25
''	Spleen NPF, .4 mg/ml	.38
Dialyzed CS + Medium NCTC 109		.19

* Complete medium: 10% chicken serum, 50% synthetic nutrient, 40% balanced saline (containing supplements where added).

† Avg values. Six flasks/series.

Summary. Chick skeletal muscle fibroblasts in monolayer cultures grow actively in homologous undialyzed serum supplemented with Medium 199. Dialysis of chicken serum removes a factor essential for growth and maintenance of these cells. The deficiency syndrome in cultures with dialyzed serum can be corrected by chicken serum dialysate, but not by inositol, cholesterol, vit. B_{12}, spleen nucleoprotein fraction, or by a more complex synthetic nutrient, NCTC 109. Proteose-peptone at 0.1% may be substituted for serum dialysate to restore full activity of whole serum.

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Received September 16, 1959. P.S.E.B.M., 1959, v102.

Influence of Dose Rate on Radiation Effect on Fertility of Female Mice. (25288)

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Studies of the variables that affect the action of radiation on cell populations may be expected to yield answers concerning both the nature of the radiation effect and the properties of cell population concerned. An understanding of these factors is of special importance in gametogenic cells, for proper interpretation of results of genetic experiments is not possible. It has long been known that, following irradiation with even a relatively low acute dose, female rodents produce only one or a limited number of litters before permanent sterility sets in, a response entirely different from that of males, which regain fertility even after high doses. The basis radiation-induction of permanent sterility in the female is the extremely rapid destruction of immature primordial oocytes, shown for the mouse by Brambell and Parkes(1) and by Murray(2), whose findings were, in general, confirmed recently by Oakberg(3). The careful quantitative studies of Mandl(4) in the rat lead to similar conclusions. (Her paper gives a review of some of the voluminous literature on radiation effects on the ovary.) It has been found that new formation of oocytes does not occur following irradiation(1,3, etc.), supporting the view that oogonia do not persist in the adult. Since the first meiotic division is not completed until shortly before ovulation which, in any case, involves only very few cells at each estrus, practically the entire gametogenic cell population of adult mouse ovary consists of primary oocytes. Diplotene is completed by 72 or 96 hours after birth(5). The oocyte nucleus then enters into a modified resting stage, named "dictyate"(6), that lasts throughout the subsequent life of the oocyte, until diakinesis occurs only a few hours prior to ovulation. Extreme radiosensitivity is usually associated with obvious activity, mitotic or otherwise, of cells. Therefore, in view of histological and cytological facts outlined, it seems, *a priori*, puzzling that the adult ovary should be so radiosensitive. Its gametogenic cell population consists of non-dividing cells, and the nuclei are in apparently uniform resting stage. It was in an attempt to contribute to an understanding of this rather unusual type of radiosensitivity exhibited by the mammalian oocyte that the present experiments were undertaken. Preliminary reports of some of the results have been published(7, 8). Various factors which might be expected to influence response to dose-rate changes could very well work in opposite directions to each other. On the one hand, lowering dose rate might, in some respects, tend to increase the sterilizing effect. Thus, for matings made at a given interval following accumulation of total dose, the lower the dose rate, the earlier and, therefore, the more sensitive the oocyte stage at which radiation was begun. Similarly, existence of some cycle of sensitivity, might result in more damage from radiation distributed in time than from a single acute dose. On the other hand, if repair of damage can occur in oocytes, lowering of dose rate should, in this respect, tend to decrease the effect of a given dose. Past fractionation and dose-rate experiments on female mice have yielded apparently conflicting results. While some investigators indicated that fractionation of dose lessens the sterilizing action of radiation(9,10), others noticed only a limited effect of changes in dose rate(11), or no effect(12). Still others claim to have found an *inverse* relation between dose rate and sterilizing action(13), *i.e.*, the lower the rate of administration of a given dose of chronic radiation, the greater the damage. It will be shown that these apparent conflicts can be explained in the light of present findings.

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* Operated by Union Carbide Nuclear Co. for U.S. Atomic Energy Comm.

Material and methods. The following procedures apply to both experiments (fractionation and continuous exposure). Females used were genetically uniform (101 x C3H) F_1 hybrid mice and were at least 6 weeks old when treatment was begun. In the 2 experiments, females were distributed randomly among various component groups. Each group was comprised of 12 females, with exception of the control group for the continuous exposure experiment, in which there were 18. The course of exposures was arranged in such a way that the various groups, within each of the 2 experiments, completed accumulation of their respective total doses on approximately the same date. Six females of each group were mated immediately following total dose accumulation, while the other 6 were mated 10 days later (corresponding control groups being mated concurrently). This was done to provide information for the intervals where there would otherwise be gaps, *i.e.*, when females would not be available for mating as a result of being pregnant. Age at time of mating ranged from 3-5 months. Each female was caged separately with a (101 x C3H) F_1 male for the rest of her life. Litters were recorded and killed on day of birth. In the fractionation experiment, females were exposed to either a single or to fractionated doses of acute X rays (dose rate about 80 r/min; 250 Kvp; 15 ma; inherent filtration 3 mm Al; H.V.L. 0.4 mm Cu; target-object distance 84 cm; exposure in individual lucite tubes on rotating 10.8-cm-high masonite scatter block; heads of animals shielded). Total doses and ways in which they were fractionated are shown in Tables I and II. There are 2 groups of controls: one "exposed" to one sham-irradiation; and one "exposed" to 30 sham-irradiations (5/week), *i.e.*, receiving as much handling as the most frequently handled irradiated group. The experiment, with controls, thus comprised 12 groups. The 144 females produced 5,810 young in 958 litters. In the continuous exposure experiment, females were exposed to radiation from a 5 curie Cs^{137} source. Exposure was continuous except for about 1-2 hours each week. Total doses† and dose rates, which were regulated by distance, are shown in Tables III and IV. The experiment comprised 14 groups. The 174 females produced 12,494 young in 1,970 litters.

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† In a preliminary abstract of this experiment(8), the total accumulated doses were reported about 16-17% higher than here. The revision was made following an accurate recheck of dosimetry.

Results. Total performance of various groups is summarized in Tables I and II for the fractionation experiment and in Tables III and IV for the continuous exposure experiment. Tables I and II also include, in addition to results of the present experiment, some unpublished data of W. L. Russell and J. S. Gower for performance of (101 x C3H) F_1 females following single acute doses of 300, 400, or 500 r (based on 59, 130, and 26 females, respectively.

Analysis of the data in terms of time elapsed following irradiation is presented in Figs. 1 and 2 for fractionation and continuous exposure experiments, respectively. In these figures, 2 parameters are plotted for

TABLE I. Litters Produced by Females Irradiated with Acute X-rays in Single or Fractionated Exposures. Mean number of litters/female and stand. errors.

Total dose (r)	Single exposure (unpublished data of W. L. Russell and J. S. Gower)	Single exposure	25-r fractions (2/wk)*	10-r fractions (5/wk)†
0		14.67 ± .56		15.17 ± .64‡
50		4.00 ± .21	9.00 ± .33	12.82 ± .69
100		2.08 ± .08	3.33 ± .25	9.67 ± .98
200		1.75 ± .13		4.17 ± .32
300	1.36 ± .06	1.42 ± .15		2.00 ± .0
400	1.14 ± .04			
500	.69 ± .12			

* Irradiated Mondays and Fridays. † Irradiated daily, Mondays through Fridays. ‡ This group of controls underwent 30 sham-irradiations. Mean No. of litters for the 2 control groups combined is 14.92 ± .42.

TABLE II. Offspring Produced by Females Irradiated with Acute X-rays in Single or Fractionated Exposures. Mean number of offspring/female and stand. errors.

Total dose (r)	Single exposure (unpublished data of W. L. Russell and J. S. Gower)	Single exposure	25-r fractions (2/wk)*	10-r fractions (5/wk)†
0		91.17 ± 2.71		91.67 ± 5.97‡
50		23.17 ± 1.48	52.25 ± 3.39	79.82 ± 5.40
100		14.08 ± .58	17.75 ± 1.74	61.83 ± 1.84
200		10.00 ± 1.02		21.00 ± 1.46
300	8.44 ± .36	7.58 ± .94		13.67 ± .70
400	5.84 ± .26			
500	1.85 ± .41			

* Irradiated Mondays and Fridays. † Irradiated daily, Mondays through Fridays. ‡ This group of controls underwent 30 sham-irradiations. Mean No. of young for the 2 control groups combined is 91.42 ± 3.20.

TABLE III. Litters Produced by Females Irradiated with Various Dose Rates of Continuous Gamma Radiation from Cs^{137}. Mean number of litters/female and stand. errors.

Total dose accumulated (r)	Dose rate (r/wk)				
	—	10.1	23.1	47.2	88.2
0	13.88 ± .37				
86		13.33 ± .73	12.08 ± .68	13.25 ± .45	13.33 ± .45
171			13.00 ± .50	11.33 ± .70	11.00 ± .37
256			11.83 ± .27	11.25 ± .51	8.42 ± .34 9.00* ± .64
345				9.25 ± .22	7.75 ± .46

* Results of pilot experiment, which preceded the experiment proper by a few months. All other groups concurrent.

TABLE IV. Offspring Produced by Females Irradiated with Various Dose Rates of Continuous Gamma Radiation from Cs^{137}. Mean number of offspring/female and stand. errors.

Total dose accumulated (r)	Dose rate (r/wk)				
	—	10.1	23.1	47.2	88.2
0	84.76 ± 3.08				
86		86.67 ± 7.15	78.08 ± 3.56	83.00 ± 3.11	86.33 ± 1.72
171			86.00 ± 3.80	73.67 ± 5.48	62.17 ± 2.73
256			72.67 ± 2.19	72.67 ± 4.32	51.75 ± 3.46 59.00* ± 6.09
345				59.08 ± 1.94	47.83 ± 2.65

* Results of pilot experiment, which preceded the experiment proper by a few months. All other groups concurrent.

each group. The top graph shows, for each time interval, percentage of females that had at least one litter in that time interval. (For the sake of brevity this parameter will be referred to, subsequently, as percentage of "productive" females.) The bottom graph represents mean size of litters born. Most females regularly mater postpartum during major portion of their reproductive life. When young are killed at birth, litters from such matings are spaced approximately 3 weeks apart, and the results have consequently been tabulated for 3-week intervals, such that, during the height of reproductive activity, almost every female had one litter in each interval. (Occasionally, synchrony broke down leading to 2 litters in one interval and none in the next. When this happened with several females in a group, the top graph takes an artificial dip not indicative of lowered performance; see, *e.g.,* 64-84 day intervals in Fig. 2B, C, D). The first interval, which starts at 19 days (*i.e.,* length of gestation period) is 24 instead of 21 days long. This has been done to include the first surviving litter from those females whose first conceived litter died as a result of the high dominant lethal incidence from matings made in the night following irradiation(14). Since, handling had apparently no effect on performance of nonirradiated animals, (Tables I and II) the 2 control groups used in the fractionation experiment have been combined for Fig. 1. The few females that died before the end of their reproductive period (one in the group that received 50 r in 10-r fractions, one in controls of continuous ex-

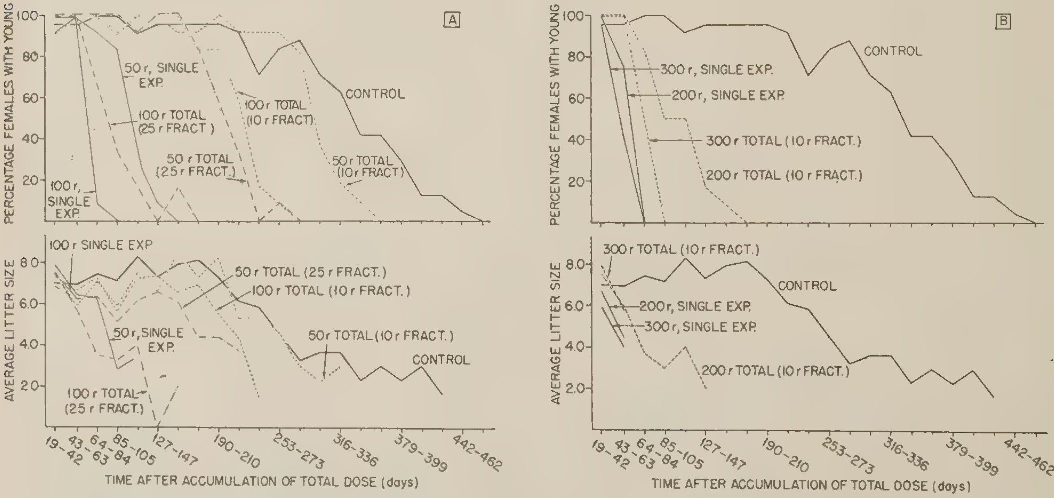

FIG. 1. Percentage of females that had at least one litter (upper graphs) and mean littersize (lower graphs) in various intervals (of 3-wk duration each) following accumulation of various total doses of X-rays, administered either in a single exposure or in fractions of 25 r or 10 r. (A) Total doses of 50 r and 100 r. (B) Total doses of 200 r and 300 r. Note: mean littersize is plotted only for intervals in which at least 10% of females had litters.

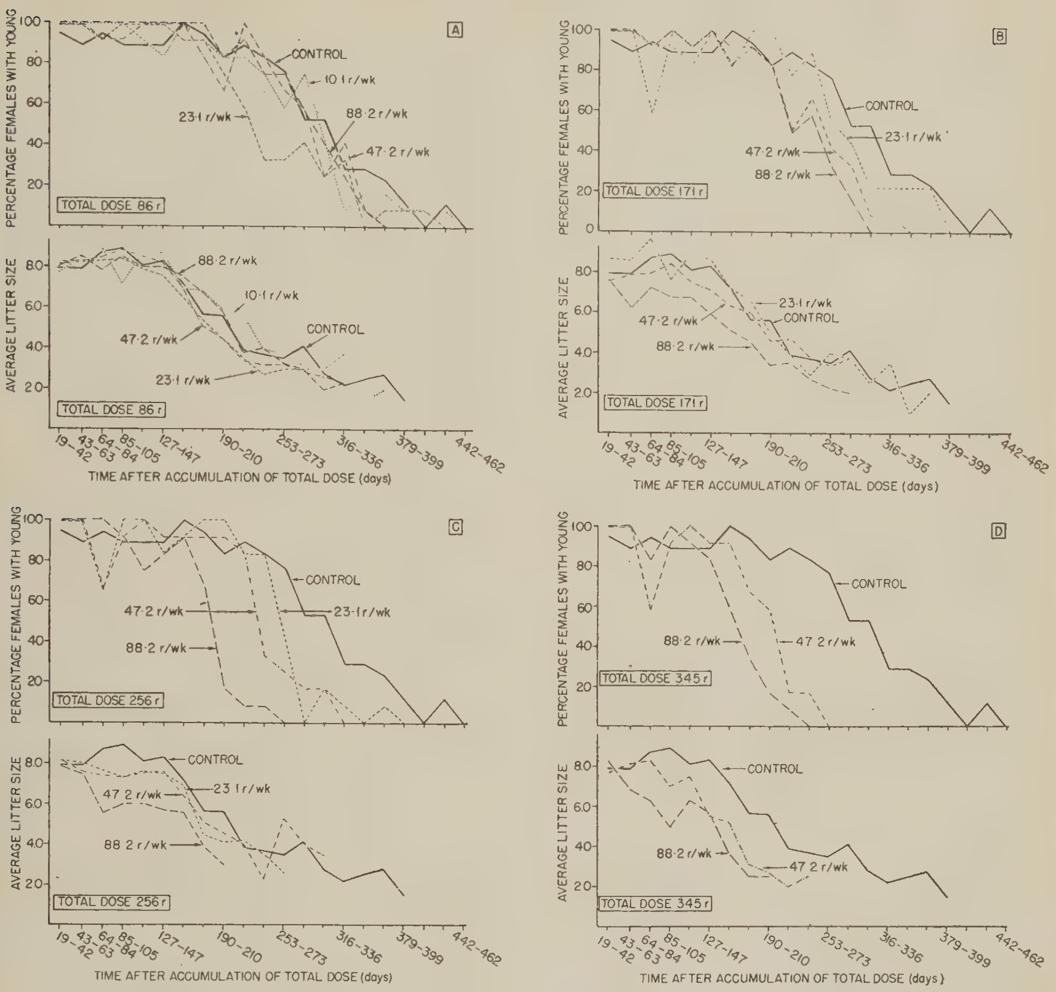

FIG. 2. Percentage of females that had at least one litter (upper graphs) and mean littersize (lower graphs) in various intervals (of 3-wk duration each) following accumulation of various total doses of continuous gamma rays, administered at dose rates of 88.2, 47.2, 23.1, or 10.1 r/wk. (A) Total accumulated dose, 86 r. (B) Total accumulated dose, 171 r. (C) Total accumulated dose, 256 r. (D) Total accumulated dose, 345 r. Note: mean littersize is plotted only for intervals in which at least 10% of females had litters.

posure experiment, and 3 in group that received 171 r at 23.1 r/wk) do not contribute to the Tables or to Fig. 3, but are included in Fig. 1 and 2 until the time of their death.

The results for both fractionation and continuous exposure experiments are summarized in Fig. 3.

A. Single, acute exposure. Even a dose as low as 50 r, when given in a single exposure, reduces performance of females drastically, *i.e.*, to one-fourth of normal (Table I and II). This is all the more striking since higher doses do not greatly increase the effect (Fig. 3). This is, of course, due to the very high sensitivity of early oocytes and relatively high radiation resistance of oocytes contained in mature follicles, as demonstrated by histological studies of the ovary (see above), and by studies of ovulated eggs during dominant lethal experiments(14). As a result of radiation resistance of mature oocytes, even females exposed to high doses usually produce one or 2 litters before becoming sterile. Thus, in the group exposed to 400 r, 98% produced at least one litter; and, even after 500 r, 62% had one or more litters.

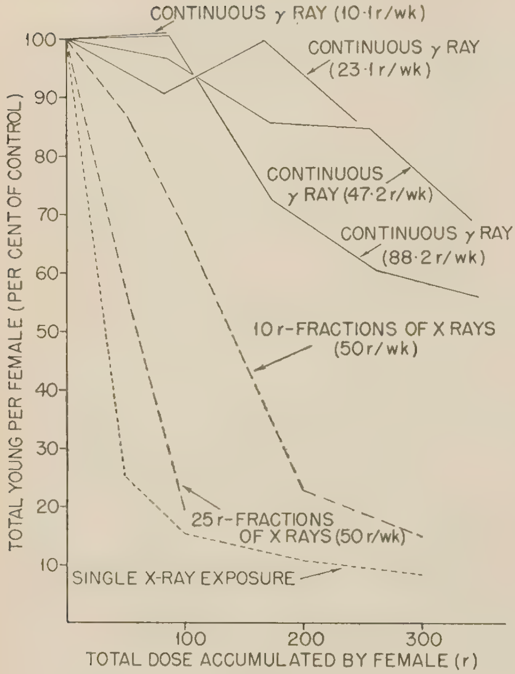

FIG. 3. Total No. of offspring produced by females following irradiation with various doses, administered in single, acute exposure, in fractionated acute, exposures, or, in continuous exposure at various dose rates; expressed as % of controls.

The dose curve for total number of young produced following a single, acute X-ray exposure (Fig. 3, lowest curve) is, thus, obviously a compound curve. The relatively level portion at higher doses represents radiation effects on resistant mature oocytes, which give rise to progeny even after doses that have killed the less mature, more sensitive cells. (It should be pointed out that most, if not all, of the slope in that portion of the curve is probably due to dominant lethals, *i.e.*, induced genetic changes leading to death of embryos(14,15) rather than to killing of oocytes.) The initial steep slope of the curve is presumably compounded of various slopes for the more sensitive stages. Thus, the dose-effect curve for most immature oocytes alone (*i.e.*, those found by Oakberg to be most quickly killed by radiation) is presumably considerably steeper even than the steepest portion of the compound curve. It might be possible, with more extensive data, to analyze the curve into its component parts, thereby determining percentage contribution to total progeny made by each oocyte stage, as well as slope of killing curve characterizing each of these stages.

The histological finding(3) of marked correlation between degree of immaturity and radiosensitivity of oocytes is well borne out by breeding data. When performance is examined in terms of time elapsed after irradiation (Fig. 1) it becomes obvious that number of litters cast does not decrease until just prior to complete sterility. On the simplest assumption, this result would indicate that increase in sensitivity with immaturity is a rather steep one.

B. Effect of fractionation. Fractionation of dose markedly reduced the damage to fertility, and that division of dose into 10-r fractions was more effective in this respect than division into 25-r fractions. At each total dose, the differences between exposure groups (single exposure *vs.* 25-r fractions, 25-r fractions *vs.* 10-r fractions) are highly significant (in all comparisons, $P << 0.0001$) for mean number of litters cast (Table I). The same is true for mean total number of young (Table II) except for one difference (namely, that between single exposure and the 25-r fraction group at 100 r total dose), which is significant only at a lower level ($P = 0.047$).

The results for total young/female may be compared in the summarizing Fig. 3. For initial steep portions of curves, the ratio of slopes is about 4 in comparison between single exposure and 10-r fractions, and about 2 for comparison between single exposure and 25-r fractions. (Note that these ratios may be underestimates, since initial portion of the curve for single, acute exposure may very well prove even steeper when dose points intermediate to 0 and 50 r are filled in.) This indicates that the sensitive, immature oocytes are capable of considerable recovery from radiation damage. As the curves flatten out at higher doses, the ratio of slopes decreases. It seems probable that the curves would approach each other even more at higher doses, since one would deal there only with the most mature oocytes, the viability of which is essentially unaffected by radiation. Whether or not some differences remain due to genetic changes (dominant lethals) induced in these cells is problematic.

When the results are examined in detail, in relation to time elapsed after irradiation (Fig. 1), it appears, for number of productive females ("percentage females with young"), that fractionation of dose is roughly equivalent to giving a lower dose in single exposure. Similarly, 10-r fractions at one dose are roughly equivalent to 25-r fractions at a lower total dose. Thus, the extra young obtained when doses are fractionated, are not the result of prolongation of a period of low-level fecundity. Instead, the period of full-scale production is extended and, as in single exposures, number of productive females does not decrease appreciably until shortly before sterility sets in. Time relations for littersize will be discussed below.

C. Effect of lowering dose rate. It may be seen, by comparing the results in Tables III and IV with those in Tables I and II, that continuously exposed females were considerably less affected in breeding performance than those which received fractionated irradiation. This is true even of the comparison between highest dose rate of continuous irradiation (88.2 r/wk) and smallest fractions of acute irradiation (5 10-r fractions/week) (Fig. 3). Groups that received a total accumulated dose of 86 r were not significantly different from controls in their performance. This is in contrast to animals receiving only 50 r in 10-r fractions which differed significantly in mean number of litters from combined controls of the fractionation experiment.

Further, in comparing different dose-rate groups within the continuous exposure experiment, it is obvious, at least at total doses above 86 r, that the lower the dose rate the smaller is the deleterious effect on fertility. Both for mean number of litters (Table III) and mean number of young (Table IV), the difference between 88.2 r/wk and 23.1 r/wk is clearly significant at total doses of 171 r and 256 r ($P = 0.001$ and $P << 0.0001$, respectively, for litters; $P << 0.0001$, at each total dose, for offspring); and the difference between 88.2 r/wk and 47.2 r/wk is clearly significant at total doses of 256 r and 345 r ($P << 0.0001$ and $P = 0.003$, respectively, for litters; $P = 0.0002$ and $P = 0.0006$, respectively, for offspring). Of the 3 remaining dose-rate comparisons at total doses above 86 r, 2 are on the borderline of significance for at least one parameter (23.1 r/wk *vs.* 47.2 r/wk at 171 r, both for number of litters, $P = 0.05$, and number of young, $P = 0.06$; 47.2 r/wk *vs.* 88.2 r/wk at 171 r for number of young, $P = 0.06$); and one (23.1 *vs.* 47.2 at 256 r), though not significant, at least goes in the expected direction.

Analysis of results of continuous exposure experiment in relation to time elapsed after irradiation (Fig. 2), presents an even clearer picture of direct correlation between dose-rate and sterilizing effect. At each total accumulated dose, above 86 r, the decline in percentage of productive females (top portions of Fig. 2B, C, D) occurs earlier the higher the dose rate. This correlation is apparent even in the few cases where totals (Tables III and IV) failed to give clearly significant differences. Even at lowest accumulated dose, 86 r (Fig. 2A), where none of the totals differed significantly from controls, there is a faint suggestion of effect, at least for the 2 highest dose rates, 88.2 r/wk and 47.2 r/wk, which slightly curtail length of reproductive period. (Note: 23.1 r/wk results at 86 r were, for some unknown reason, temporarily out of line with all others; but the group rallied and went on breeding almost as long as controls.) In all groups, production, in terms of number of litters cast, was at maximum level until the time of decline that ends in sterility. This decline is in all cases somewhat steeper than the decline due to normal ageing (controls).

Analysis with respect to littersize (bottom portions of Figs. 1A, B; 2A, B, C, D) is not nearly as clear as that with respect to percentage of productive females. It appears, at first glance, that a great many irradiated groups are characterized by depressed littersizes through the major part of their breeding period. However, the following considerations make it questionable that this is an effect additional to the one already discussed. Thus, when decline in littersize is regarded in relation to decline in productive females (in controls the former precedes the latter), the littersize depression in each case is to be expected in terms of impending sterility. However, the total situation is undoubtedly considerably more complex. Thus, littersize is affected not only by number of oocytes ovulated, and this, under certain circumstances, may actually be *in*creased by radiation(15), but also by genetic changes induced in these oocytes that lead to death of embryos (dominant lethals) (14,15).

Discussion. That a lowering of dose rate decreases sterilizing action of a given dose of radiation has been clearly demonstrated both within the fractionation experiment and within the continuous exposure experiment. Comparison *between* experiments shows that continuous exposure at the rate of about 50 r/wk (47.2 r/wk) of gamma rays is several times less sterilizing than exposure to five 10-r fractions of X rays/week (Fig. 3). This difference is very much greater than might be explained on the basis of radiation quality alone (gamma *vs.* X rays). Thus, comparison between experiments strengthens the conclusion reached on the basis of each experiment alone.

In view of this very clear-cut dose-rate effect, it is necessary to re-examine reports in the opposite direction from that found here. It can be shown that past experiments which are in apparent disagreement with the present results, used methods of testing which were not suitable for revealing what is really the characteristic effect of radiation on female fertility, namely, shortening of breeding period.

Thus, size of first litter born following accumulation of total dose, was used as the basis for the conclusion reached by H. and M. Langendorff(12) that there is no effect of fractionation. It was used as 1 of 2 criteria of effect by Neary, Munson, and Mole(13), who report an inverse relation between dose rate and damage. Similarly, most results of Deringer, Heston, and Lorenz(11) are based on first litters; and although their comparison between acute and chronic exposure is in agreement with the present results, the authors "failed to demonstrate any effect of dose administration" when comparing their various chronic-irradiation series with each other. As is obvious from the present paper, differences in effects of various dose rates are not apparent from the first litter following irradiation. Decrease in size of first litter is probably primarily due to dominant lethals and not to decreased number of ovulations(14).

Another complication that may obscure true dose-rate effect is age of females. Unless adequate controls are used, or unless special precautions are taken to terminate radiation exposures at the same age in groups being compared, it is obvious that the lower the dose rate, the more advanced in age, and thus the less productive from natural causes, are the females when tested for fertility. This consideration must be applied to results of Deringer *et al.* and may even explain the inverse relation between dose rate and damage reported by Neary *et al.,* some of whose females were irradiated for almost 9 months. As a matter of fact, among experiments of the latter authors, there is one, for which controls were available, which seems to support this view.

Neary *et al.* suggested as possible explanation for their results that radiation sensitivity of females may increase with age. Their small, supplementary experiment on this point cannot be considered conclusive because of inadequate controls. Indeed, it seems more likely that their results are a consequence of unsatisfactory methods of testing discussed above. Another factor may also have added confusion: after long-continued irradiation, already the first mating (as well as all subsequent ones) must utilize oocytes that had received some of their dose in the highly sensitive early stages; after a shorter-term exposure, such oocytes would not be used until later matings. By either criteria used by Neary *et al.,* this would result in apparently greater damage from lower dose rates. Whether or not such a factor has played a role in results of Neary *et al.,* it must have been of minor importance in the present data, since it was obviously outweighed by factors that decrease oocyte damage as dose rate is decreased.

In the light of preceding discussion, it is clear that results of other workers are not in major conflict with the present data. Because of measures of effect chosen, the 3 experiments discussed(11-13) failed to reveal what the present data show clearly, namely, that the characteristic effect of radiation on female fertility is a shortening of the breeding period, and that this effect is very strongly dependent on dose rate.

The most plausible explanation of direct relation between dose rate and damage to oocytes is that some repair is occurring. The mechanism by which radiation kills oocytes is not known. Since the cells do not divide, chromosome imbalance cannot be the cause of death. Oakberg(3) finds the early oocytes to be killed very rapidly. Our experiments, reported elsewhere(16), indicate that the sensitive system, whatever its nature, is not markedly protected by hypoxic conditions during irradiation.

Summary. 1) In an attempt to contribute to an understanding of unusual radiation sensitivity exhibited by the mammalian oocyte, a non-dividing cell, fertility experiments were carried out to determine the effect of dose rate (fractionation, different intensities of continuous radiation). 2) Fractionation markedly reduced damage to fertility; and division of dose into 10-r fractions was more effective in this respect than division into 25-r fractions. Continuously exposed females were even less affected in breeding performance than females which had received fractionated irradiation, and the lower the dose rate the smaller was the deleterious effect on fertility. In all groups, production, in terms of number of females casting litters, remained at maximum level until beginning of a sudden steep decline that ended in sterility. It is for this reason that some experiments of other investigators, who have measured performance only in terms of the first postirradiation litter, have failed to show similar dose-rate effects. 3) The results indicate that some repair of radiation damage to oocytes can occur, and that repair is greater at lower dose rates.

The authors are most grateful to Dr. Oakberg for discussing with them his unpublished findings on radiation effects on the ovary.

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Received June 15, 1959. P.S.E.B.M., 1959, v102.

Comparison of Physostigmine and Amphetamine in Antagonizing the EEG Effects of CNS Depressants.* (25289)

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Many investigators have shown in animals that cholinergic drugs activate EEG by exciting subcortical centers(1-3). Other experiments reveal that adrenergic drugs also induce EEG activation by acting on the same areas of the brain(2-5). This indicates that the reticular activating system(6) contains both cholinergic and adrenergic sensitive neurons. The finding that chlorpromazine will block EEG action of amphetamine but not that of physostigmine(7) suggests that chlorpromazine inhibits adrenergic sensitive neurons but not cholinergic ones. It seemed of interest, to ascertain whether other neurosedatives share this property of chlorpromazine and to further study the effectiveness of physostigmine to antagonize EEG effects of CNS depressants.

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* This investigation supported by U.S.P.H.S. Senior Research Fellowship grant.

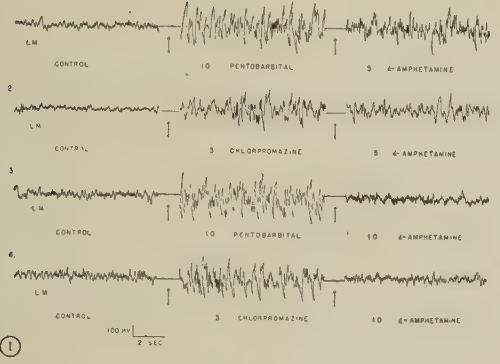
Methods. Unanesthetized albino rabbits weighing 1.7 to 3.5 kg were employed. Gross behavioral and electroencephalographic changes were observed simultaneously. Electroencephalograms were obtained(3) by inserting steel electrodes into the cranium over the right motor, left motor, left limbic, and left occipital areas of the cortex and recorded by a Grass Polygraph. For brevity, only tracings from the left motor area will be illustrated. Blood pressure was recorded from one femoral artery by mercury manometer and the respiratory rate as well as other physiological phenomena were observed. Drugs in this study were chlorpromazine HCl, pentobarbital Na, meprobamate, phenaglycodol, physostigmine salicylate, and *d*-amphetamine sulfate. Meprobamate was administered as 2.5% supersaturated solution cooled to about 45°C before injecting. Phenaglycodol was dissolved in 50% polyethylene glycol 400 vehicle. Other drugs were dissolved in distilled water or 0.9% saline. All were injected into marginal ear vein, and those that produce EEG synchronization were usually administered first. Five to 10 minutes subsequently a single injection of either physostigmine or *d*-amphetamine was made. Doses of drugs are shown in Table I. In another series of animals, physostigmine or *d*-amphetamine was administered alone and their effects noted.

TABLE I. EEG Changes Produced by Physostigmine and *d*-amphetamine on the Sleep Pattern Induced by Pentobarbital, Chlorpromazine, Phenaglycodol, and Meprobamate.

Neurosedative		Physostigmine and *d*-amphetamine		
Drug	Dose, mg/kg	Drug	Dose, mg/kg	No. activated per total
Pentobarbital	5	Amph.	1–2	0/3
		”	3–4	2/2
		Phys.	.1–.2	2/2
	10–20	Amph.	2–7	2/10
		”	10–20	7/8
		Phys.	.2–.3	14/18
Chlorpromazine	1–2	Amph.	3–5	4/9
	3–5	”	5	2/6
		”	10	3/3
	5–10	Phys.	.1–.2	8/8
Phenaglycodol	25–50	Amph.	1–3	0/4
		”	5	3/3
		Phys.	.1–.2	4/4
	60–80	Amph.	2–4	1/4
		Phys.	.1–.2	2/2
Meprobamate	40–70	Amph.	3–5	5/7
	80–140	”	2–3	0/6
		”	4–10	6/6
	100	Phys.	.2	3/3

Results. EEG and behavioral effects of neurosedatives. Activation EEG pattern was manifest in control records of most animals (Fig. 1, 2). All CNS depressants changed this alert tracing to a synchronous one (Fig. 2). Gross behavioral effects of these drugs were similar, since, as the dose increased, signs of depression became progressively more marked. Pentobarbital was more predictable in this regard than were other CNS depressants studied but even with this agent variations occurred from animal to animal. A few animals were given 25-30 mg/kg doses but an isopotential EEG resulted. They are, therefore, not included in Table I. Occasionally a low dose of chlorpromazine (1-2 mg/kg) would induce an EEG synchronization without concomitantly causing flaccid paralysis observed with higher doses (3-10 mg/kg) and in 2 animals given 60 mg/kg of meprobamate, no behavioral or EEG changes were seen until an additional 40 mg/kg was injected. These descriptions illustrate variations in response occasionally observed. There was no correlation between central effects of these drugs and changes in blood pressure nor were these changes significant enough to induce EEG changes in the rabbit(8,9). Furthermore, subsequent administration of amphetamine or physostigmine produced changes in blood pressure and respiration independent of EEG effects observed.

Amphetamine. Behavioral and EEG antagonism to effects of neurosedatives. When given alone, small doses (0.5 mg/kg) of *d*-



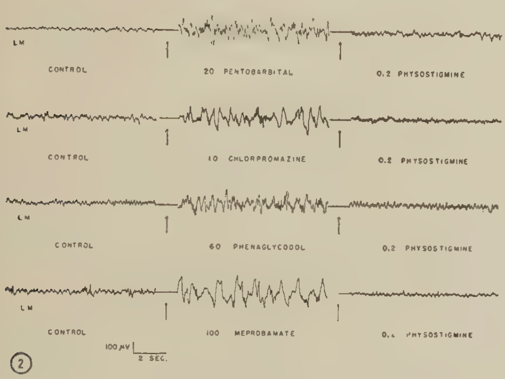


FIG. 1. Tracings 1 and 2 show that both pentobarbital and chlorpromazine can abolish the usual EEG effect of a 5 mg/kg dose of *d*-amphetamine. Tracings 3 and 4 show, however, that in higher doses amphetamine can reverse the EEG pattern induced by these agents. Numbers below records are mg/kg inj. Leads: left motor cortex (LM).

FIG. 2. Fig. shows that physostigmine in small doses (right tracings) can antagonize the EEG effects of high doses of pentobarbital, chlorpromazine, phenaglycodol and meprobamate (center tracings). Numbers below records are mg/kg inj. Leads: left motor cortex (LM).

amphetamine increased irritability of animals to stimuli, and changed a predominantly synchronous control EEG tracing in 7 to an alert one and in 3 mg/kg doses or more produced hyperactivity. Neurosedatives, in doses employed, prevented EEG (Fig. 1, Table I) and behavioral effects of small doses of amphetamine. Amphetamine if given in large enough doses, however, antagonized the EEG effect of CNS depressants (Fig. 1) and concomitantly awakened the animal. The dose of amphetamine necessary to reverse central effects of CNS depressants, paralleled the dose of the latter given (Table I). This was most evident with pentobarbital, which in higher doses increased the dose of amphetamine necessary to produce EEG activation from 20 to 40 times above that required in animals given amphetamine alone.

Physostigmine. Behavioral effects and antagonism of EEG effects of neurosedatives. When physostigmine was given alone in 0.1 mg/kg doses, it evoked EEG activation pattern in 6 of 7 rabbits which predominantly manifested a synchronous EEG control record, though otherwise they looked normal. Smaller doses produced this EEG change less consistently. In low doses (0.1 to 0.2 mg/kg) physostigmine rarely (15% of animals) produced peripheral effects such as fasciculation. In contrast to amphetamine, physostigmine (Fig. 2) in low doses antagonized the EEG patterns produced by high doses of meprobamate, phenaglycodol, chlorpromazine and pentobarbital. In some pentobarbital injected animals an additional injection was necessary to completely reverse the EEG synchronization. Physostigmine slightly elevated muscle activity in animals previously given meprobamate, phenaglycodol, chlorpromazine and low doses of pentobarbital although unlike amphetamine never provoked hyperactivity. Following higher doses of pentobarbital (15 to 20 mg/kg), physostigmine did not produce overt effects though EEG activation pattern was present for approximately 30 minutes.

Discussion. Either physostigmine or amphetamine (in sufficient amounts) antagonized the synchronous EEG pattern produced by meprobamate, phenaglycodol, chlorpromazine and pentobarbital in rabbits. However, when given after CNS depressants, amphetamine usually produced significant behavioral effects concomittant with EEG arousal, whereas physostigmine evoked EEG activation without necessarily inducing behavioral changes. Apparently physostigmine can excite corticipetal pathways of the reticular activating system(6) with little or no effect on lower parts of the neuraxis. Similarly, physostigmine does not induce signs of behavioral excitement (e.g., struggling) in cerveau isolé animals whereas amphetamine does (3). Interestingly, the physostigmine antagonists atropine and scopolamine have the opposite effect in rabbits; they block corticipetal activation pathways but not centrifugal ones (which provoke motor responses (10)).

Our findings suggest that EEG alerting mechanism is more sensitive to physostigmine than to amphetamine. Thus, in high doses, the neurosedatives increased the minimal dose of amphetamine necessary to produce its usual EEG effect by at least 4 times and with pentobarbital from 20 to 40 times (Fig. 1). In contrast, physostigmine in low doses antagonize the electrocortical effects of CNS depressants (Fig. 2). Such observations support the conclusion of Rinaldi and Himwich(1) that the reticular formation EEG activating system is fundamentally cholinergic in rabbits. The fact that low doses of atropine will block the EEG effect of adrenergic drugs but not that of physostigmine(3,11) further supports that conclusion. Higher doses of atropine will, however, prevent the EEG arousal caused by physostigmine(3,11), DFP and Ach(9).

EEG and behavioral antagonism between i.v. administered amphetamine and pentobarbital is in agreement with the use of the former as an analeptic in barbiturate poisoning(12) but is in contrast to the findings of Bradley and Elkes in cats(11). Moreover, these investigators did not find that physostigmine antagonized the EEG synchrony induced by pentobarbital. Their experiments differed from ours, however, in dosage and, in one instance, the kind of barbiturate employed and also in the form of amphetamine, route of administration (i.p.) and species employed. The observation that EEG effects of meprobamate(13) and chlorpromazine(7) can be reversed by physostigmine has been confirmed and extended to include higher doses of these drugs. The report(7) that chlorpromazine can block the EEG effect of amphetamine was also confirmed but our findings further reveal that this phenomenon is dose dependent since in higher doses amphetamine will break through this blockade. In this regard chlorpromazine resembles pentobarbital (Fig. 1).

Since all of CNS depressants (especially pentobarbital) reduced effectiveness of amphetamine to activate the EEG alerting mechanism, it seems unlikely that the property of chlorpromazine to block central effects of epinephrine(4,14) and amphetamine(7) is related to its peripheral adrenolytic action as suggested by several workers(4,7). Also it is reported that atropine can block EEG activation normally produced by amphetamine(3, 11) and other adrenergic drugs including epinephrine(3) so that chlorpromazine is not unique in this regard. Lastly, both atropine (1,10) and chlorpromazine(8,14) can abolish EEG activation resulting from direct stimulation of the midbrain reticular formation. The last cited workers(14) concluded that their electrophysiological observations did not support the interpretation of Hiebel *et al.*(4) that the blockade of the central excitatory effects of epinephrine account for the action of chlorpromazine. Thus experiments other than those based on EEG responses are apparently necessary to define adequately whether a drug acts as an adrenolytic agent centrally.

Summary. 1) Pentobarbital, chlorpromazine, phenaglycodol and meprobamate when injected i.v. into rabbits produced a synchronous (high voltage low frequency) EEG pattern. Physostigmine or *d*-amphetamine, in suitable doses, antagonized this pattern and evoked an alert (low voltage high frequency) EEG tracing. The dose of amphetamine necessary to antagonize synchronous EEG record generally paralleled the dose of neurosedative administered, especially in the case of chlorpromazine and pentobarbital. In contrast, low doses of physostigmine usually exerted its EEG effect regardless of dose of CNS depressant employed. This indicates that the EEG activation mechanism is more sensitive to cholinergic than to adrenergic drugs and supports the conclusion of several authors that the EEG alerting mechanism is fundamentally cholinergic in rabbits. 2) The evidence indicates that the EEG effects of chlorpromazine are not necessarily related to its peripheral adrenolytic actions.

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Received June 15, 1959. P.S.E.B.M., 1959, v102.

Effect of Microbial Antigens on Irradiation Mortality in Mice.* (25290)

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Boivin-type endotoxins from *Proteus morganii* decrease mortality in x-irradiated mice (1,2). It is also known that endotoxins from *Salmonella typhosa, Escherichia coli,* and *Serratia marcescens* increase the survival of irradiated mice, rats, and hamsters(3,4). Since the chemically isolated lipopolysaccharide substances called endotoxins or toxic somatic antigens are thermostable antigenic components associated with the bacterial surface, bacterial vaccines and boiled culture supernatants, both of which would be expected to contain these somatic antigens, should also be effective in decreasing irradiation mortality. For this reason, the present preliminary investigation was initiated. We have found that mortality is definitely decreased by injection of Gram-negative antigens. Gram-positive antigens, however, appear ineffective in this respect. Other attempts to decrease mortality by means of bacterial antigens other than purified endotoxins have been unsuccessful (5).

Materials and methods. Thermostable antigens were prepared from *Klebsiella pneumoniae, Proteus morganii, Escherichia coli,* and an α hemolytic Streptococcus by heating saline suspensions of each organism for 1 hr in a boiling water bath. Following clarification, toxicity tests were performed on each of the sterile antigen solutions using non-irradiated mice. Groups of mice were injected intravenously with 0.1 ml of a sublethal dilution of each antigen 24 hr before irradiation. The following commercial (Parke, Davis) antigens were used: Streptococcus (Bio. 327), each ml containing saline extractable substances from 2×10^8 hemolytic and non-hemolytic Streptococci; Staphylococcus (Bio. 385), each ml containing soluble antigens from 1×10^9 *Staphylococcus aureus* and *Staphylococcus albus* mixed with Staphylococcus toxoid; Typhoid-Paratyphoid vaccine (Bio. 446), each ml containing 1×10^9 killed *Sal. typhosa,* 250×10^6 *Sal. paratyphi,* and 250×10^6 *Sal. Schottmulleri.* All of these antigens were used undiluted and given intravenously in a volume of 0.1 ml. A concentrated saline suspension of zymosan was heated in a boiling water bath for 1 hr, diluted as required with buffered saline (pH 7.0), and injected intravenously in a volume of 0.2 ml. Zymosan was obtained from Immunological Specialties, Los Angeles. Total body x-irradiation was delivered from a 200 kv Picker-Waite X-Ray therapy machine operated at 20 ma and a distance of 20 cm. Added filtration consisted of 0.5 mm Cu and 1 mm Al. Mice used were

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* Part of data from dissertation submitted in partial fulfillment of requirements for degree of Doctor of Philosophy. This work supported in part by contract AT(30-1)-2018 between the Atomic Energy Comm. and Brown University, and USPHS grant C-592.

† Predoctoral Trainee of U. S. Nat. Cancer Inst.

TABLE I. Survival of Mice Treated with Antigen 24 Hr before Irradiation with 700 r.

Antigen	30 day mortality	%	Level of significance of mortality reduction
Klebsiella (1:10)	17/30*	56	P <.001
Proteus (1:100)	14/30	46	"
Escherichia "	9/18	50	"
α Streptococcus	28/30	93	P >.05
Zymosan 300 γ	15/18	83	"
" 1000 γ	16/30	53	P <.001
Streptococcus	18/18	100	
Staphylococcus	18/18	"	
TAB	1/18	5.5	"
Controls	26/26	100	

* No. dead/total irradiated.

BUB females, 60-90 days old weighing approximately 25-30 g. Animals were kept in temperature regulated rooms and given tap water and Purina Lab Chow *ad libitum*.

Results. Irradiation mortality is decreased from 100% to approximately 50% in animals injected with heated Klebsiella, Proteus, or Escherichia antigens 24 hr before irradiation with 700 r (Table I). The interval of 24 hr between injection and irradiation was chosen for antigen screening since this interval had proven most effective with Boivin-type endotoxins from *Proteus morganii*. The best protection at 700 r is afforded by Typhoid-Paratyphoid vaccine (TAB), mortaltiy being reduced by 94% by a single injection 24 hr before irradiation.

Mortality is not significantly reduced, however, among animals injected with antigens from Gram-positive organisms. A dose of 300 γ of zymosan also fails to decrease mortality, but 1000 γ decreases mortality to approximately the same extent as do heated bacterial antigens. Since TAB vaccine proved most effective at 700 r, it was tested at 600 r, an irradiation dose at which post-irradiation treatment with endotoxin is effective (Table II). No deaths are observed among animals given a single injection 24 hr before irradiation. Post-irraditiaon treatment is less effective, but mortality is decreased by more than 64%. Multiple injections of TAB vaccine, given 7, 4, and 1 day before 600 r, significantly decrease mortality but are less effective than a single pre-irradiation injection ($P < .001$).

Long-term survival was recorded among TAB-treated mice irradiated with 600 r. In one experiment involving 43 such animals (alive at 30 days) 11 died within the succeeding 188 days (27%). In another experiment, 67 TAB-treated animals were alive at 30 days and 11 of these died in the succeeding 117 days (16%). A total of 11 non-treated irradiation controls were available for study (controls from both experiments) and in this group no deaths have been observed in the ensuing periods.

Discussion. Thermostable antigens from Klebsiella, Proteus, or Escherichia decrease irradiation mortality in mice. The active component is undoubtedly the endotoxin or somatic antigens from the respective organisms since these effects can be produced using Boivin-type endotoxins or other so-called purified somatic antigens or endotoxins(1-4). The extent of mortality reduction following treatment with heated Gram-negative antigens is less than that observed using TAB vaccine or that previously observed using Boivin-type endotoxin. This may, however, be a result of antigen dosage rather than a differential effectiveness of certain antigens, since higher doses of endotoxin appear less effective than lower doses(4). These doses of heated Gram-negative antigens were rather high since several injected animals died within 4 days, probably from the lethal effects of the endotoxins.

TAB vaccine is highly effective in reducing irradiation mortality and appears as effective as Boivin-type endotoxin in this respect. In fact, at 24 hr after irradiation with 600 r, injection of TAB vaccine results in a greater reduction of mortality (69%) than does an injection of *Proteus morganii* endotoxin (29%). Rowen *et al.*, studying the effects of

TABLE II. Increased Survival of Irradiated Mice (600 r) Treated with Typhoid-Paratyphoid Vaccine.

Time of inj.	30 day mortality	%	Level of significance of mortality reduction
7, 4, 1 day before irrad.	7/24*	29	P <.02
24 hr " "	0/36	0	P <.001
2 hr after "	8/36	22	"
24 hr " "	7/36	19	"
Controls	19/30	63	

* No. dead/total irradiated.

bacterial antigens on irradiation mortality, found mortality to be increased among mice injected with typhoid vaccine 5 days before irradiation(5). Although we have not tested the efficacy of TAB vaccine at this interval before irradiation, we previously found *Proteus morganii* endotoxin to be ineffective if given 4 days before irradiation. Rowen's negative results are probably a function of time of injection.

The finding reported here that multiple injections of TAB vaccine decrease mortality is at variance with observations of Smith *et al.* who reported no increased survival of mice given multiple injections of typhoid endotoxin(4). These differing results may be explained by the use of different antigen dosages. It appears, however, that mice become somewhat refractory to the protective effects of TAB vaccine or endotoxin following multiple injections. An interesting observation, based, however, on rather small numbers, is that after 30 days no deaths occurred among irradiation control animals, whereas considerable mortality was observed during this period among TAB treated animals. The protection afforded by TAB vaccine appears to be only temporary.

Although conflicting reports are present in the literature concerning the effect of zymosan on irradiation mortality(6,7), protective effects have recently been reported(8,9), and the results reported here confirm these positive results. Zymosan and somatic antigens or endotoxins have many properties in common. Both have a high polysaccharide content(10, 11), both induce tumor necrosis(12,13), both have an adjuvant effect on antibody production(14,15), and appropriate doses of both elevate properden titers(16). Although substances from some Gram-positive organisms have certain endotoxic properties(17), soluble antigens from Staphylococci and Streptococci are ineffective in decreasing irradiation mortality.

Summary. Mortality is reduced by approximately 50% in mice injected with zymosan (1000 γ) or with thermostable antigens from *Proteus morganii, Klebsiella pneumoniae,* or *Escherichia coli.* Soluble antigens from Gram-positive organisms, however, are ineffective. The greatest reduction of mortality is obtained by means of a Typhoid-Paratyphoid vaccine.

The authors wish to acknowledge Dr. Milford H. Hatch for advice and suggestions during preparation of manuscript.

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Received June 25, 1959. P.S.E.B.M., 1959, v102.

Modification of Bactericidal Effects of Human Sera.* (25291)

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We employed antigenically similar strains of *Shigella dysenteriae* with inherent differences in their susceptibility to bactericidal effects of so-called normal human serum(1) to study modifying effects of carbohydrates and amino acids on serum susceptibility of bacteria *in vitro*. Changes in bacterial sensitivity to serum following changes in conditions affecting bacterial metabolism have been noted previously(2,3). In our studies we became aware of such modifications when, in studies on serum spheroplasts(4), pregrowth of bacteria in different broth media resulted in significant alteration of their subsequent susceptibility to antibody- and complement-containing human serum. This altered susceptibility represented merely a phenotypic modification without genetic changes, and altered sensitivity was also obtained when the bactericidal system was directly supplemented with different concentrations of various growth media. The latter condition, therefore, was utilized as a routine test system for measuring modifying effects of non-bactericidal and non-bacteriostatic concentrations of various supplements on serum susceptibility of *Sh. dysenteriae* and *Escherichia coli.*

Methods. Previously described(1,4) serum-resistant (S_1) and serum-sensitive (S_3) strains of *Sh. dysenteriae,* and a serum-resistant (SPI) and a serum-sensitive strain of *Escherichia coli,* were employed. Bactericidal serum effects were tested by determination of survivors following exposure of approximately 3 to 4,000 bacteria/ml to appropriately diluted serum in ABA buffer ± supplement for 60 minutes at 37°C(1). All tests were made at least in triplicate and plated in duplicate; as a rule standard errors approximated 5 to 10% of survival values(1). Preliminary tests employed determinations of the 50% endpoint in serial dilution tests, conducted in cup-like depressions of plastic trays (Disposo trays, Linbro Chemical Corp., New Haven, Conn.) where bactericidal reactions were terminated by addition of 2 ml of melted agar to 0.5 ml of a 60-minutes-old bacteria-serum suspension(5). All supplements were dissolved in saline, pH adjusted to 6.8, and added to equal volume of ABA buffer + serum. Serum from one human donor was used throughout; this serum apparently contained low levels of specific antibodies, since absorption with homologous organisms significantly reduced its bactericidal activity(4).

Results. Effects of addition of broth on serum susceptibility of 2 strains of *Shigella* are illustrated in Table I; high concentrations of broth protected bacteria from bactericidal serum effects, whereas low concentrations (<1:1000) enhanced their susceptibility. Supplementation with nutrient broth (Difco) and penassay broth (Difco) gave comparable results.

To test involvement of specific broth constituents in these modifications, the influence of glucose and certain other carbohydrates, added directly to the bactericidal serum system, was tested. Table II shows that 1 mg of glucose/ml significantly enhanced bacterial sensitivity, whereas other carbohydrates, in-

TABLE I. Effects of Broth on Serum Susceptibility of *Shigella dysenteriae,* Strains S_1 and S_3.

		% survivors after 60′ exposure	
Broth added	Dilution	S_1	S_3
Tryptose	1:1	88	102
	1:10	36	32
	1:100	9	19
	1:1000	40	37
Casein hydrol.	1:1	96	108
	1:10	40	29
	1:100	12	9
	1:1000	47	36
Brain-heart	1:1	101	82
	1:10	53	35
	1:100	9	10
	1:1000	30	36
None (serum control)		42	31
	Serum dil.:	1:2.5	1:10
	Inoc. size:	3070/ml	3550/ml

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* Supported by USPHS grant.

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TABLE II. Effects of Carbohydrates on Serum Susceptibility of *Shigella dysenteriae*, Strains S_1 and S_3.

Sugar added	Conc., mg/ml	% survivors after 60′ exposure S_1	% survivors after 60′ exposure S_3
Glucose	10	39	28
	1	7	10
	.1	26	36
Galactose	10	42	40
	1	22	25
	.1	30	38
Lactose	10	36	40
	1	40	43
	.1	31	53
None (serum control)		34	45
	Serum dil.:	1:2.5	1:10
	Inoc. size:	3720/ml	3550/ml

cluding galactose, lactose, and also ribose, sucrose, and soluble starch, had no significant effects. Since glucose is the most readily utilized carbohydrate for organisms used, these observations were the first to suggest possible relationships between metabolism and bacterial sensitivity to serum.

This relationship was further indicated by the finding that presence of amino acids known to play a prominent role in metabolism of these bacteria similarly enhanced their sensitivity to bactericidal serum effects. Following determination of the range of nontoxic concentrations of 21 different amino acids, appropriate amounts of dl-forms of these acids were added to the bactericidal serum systems. Table III illustrates the different types of modified serum effects obtained with strain S_1 in the presence of certain amino acids; comparable data were obtained with strain S_3 and *E. coli* strains.

Since the most striking enhancement of bactericidal effects occurred in the presence of glucose and of those amino acids known to stimulate metabolic activity of bacteria here employed, it was believed that bacterial lysis resulting after injury to bacterial wall components may be dependent upon the extent of metabolism of injured cells. In other words, the previously demonstrated interference of serum factors with the integrity of bacterial wall components(4) may not suffice to cause bacterial lysis. The latter may occur only if wall-damaged bacteria "grow out of their damaged walls." If these considerations were correct, interference with metabolism would be expected to prevent bacterial lysis and killing even in presence of damaged walls. This was the case. As shown in Table IV, addition of 2 amino acid analogues, 5-methyl tryptophan, and fluorophenylalanine, interfered with bacterial killing in the presence of usually bactericidal concentrations of serum. Similarly, in studies with *E. coli* strains, it was demonstrated that presence of chloramphenicol (Table V), or of the just cited analogues, interfered with serum lysis of bacteria. Inhibitory effects of analogues or of chloramphenicol were absent, or significantly reduced, when bacteria were exposed to these compounds prior to treatment with unsupplemented serum. These data thus strongly supported the existence of a fundamental relationship between extent of bacterial susceptibility to serum and extent of bacterial metabolism subsequent to, or at time of, wall damage.

Two somewhat different types of protective effects of high concentrations of certain basic amino acids (Table III) were observed; both of these may be interpreted conditionally as

TABLE III. Effects of Amino Acids on Serum Susceptibility of *Shigella dysenteriae*, Strain S_1.

Amino acids added	% survivors after 60′ exposure in presence of: 1 mg/ml	% survivors after 60′ exposure in presence of: 10 mg/ml
None (serum control)	38	38
Enhancing		
dl-alanine	10	17
-aspartic acid	11	6
-glutamic "	10	8
-asparagine	11	17
Glycine	23	17
DAP	15	33
Protecting		
dl-cysteine	34	72
-ornithine	44	78
-arginine	53	86
Concentration-dependent		
dl-lysine	17	84
-histidine	13	57
Ineffective		
dl-serine		
-threonine		
-valine		
-methionine		
-leucine		
dl-citrulline		
-phenylalanine		
-tyrosine		
-proline		
-tryptophane		

Serum dil.: 1:2.5. Inoc. size: 3260/ml.

TABLE IV. Influence of 2 Amino Acid Analogues on Susceptibility of *Shigella dysenteriae*, Strains S_1 and S_3, to Bactericidal Serum Effects.

		% survivors after 60′ of exposure			
		S_1		S_3	
Compound added	Conc., mg/ml	Inhibitor control	Inhibitor + serum	Inhibitor control	Inhibitor + serum
5-methyl dl-tryptophan	1	94	61	87	77
	5	110	78	97	90
dl-p-fluorophenylalanine	1	91	57	103	62
	5	100	70	85	84
None (serum control)	0	92	32	97	40
	Serum dil.:	1:2.5		1:10	
	Inoc. size:	3420/ml		3480/ml	

involving an interference with attachment of serum bactericidins to sites on the bacterial surface. The first type of effect (upper part of Table VI) was dissociable; this means that following 2 hours incubation of bacteria with so-called basic amino acids in buffer, the protective effect was removed by simple washing with saline prior to serum treatment. In contrast, if nutrients, such as tryptose broth, were present during pretreatment with basic amino acids (lower part of Table VI), the protective effect was *not* dissociable by washing.

TABLE V. The Effect of Chloramphenicol on Serum Susceptibility of *Escherichia coli* Strains.

	Coli SP1		Coli S	
	% survivors after 60′ exposure to:			
Chloramphenicol, μg/ml	S-F*	S-C*	S-F	S-C
10	92	46	95	59
5	99	82	101	84
1	103	72	116	68
.5	95	47	96	43
.1	84	37	92	39
.05	103	34	94	52
.01	92	33	104	37
—	103	34	99	39
Serum dil.:	1:1.5		1:10	
Inoc. size:	2510/ml		3110/ml	

* S-F, serum-free suspensions; S-C, serum-containing suspensions.

Absence of any irreversible *direct* effects of basic amino acids on bactericidal serum itself was indicated by finding that sera first exposed to amino acids for various periods of time and then dialyzed against saline did not show any altered bactericidal effects.

The protective effects of basic amino acids occurred only in pH ranges of 6.8 and above, and disappeared in more acid pH ranges. A comparison of related serum-resistant and serum-sensitive strains revealed that serum-sensitive strains had a requirement for higher concentrations of basic amino acids, to achieve the same degree of protection obtainable by lower concentrations with serum-resistant strains.

When mixtures containing both enhancing and protective amino acids were added to serum, the protective effects always counteracted any enhancing effects (Table VII). Further, combinations of different enhancing amino acids were more effective in increasing bacterial susceptibility to serum than comparable amounts of single amino acids (Table VII). Finally, non-bactericidal concentrations of dipeptides, added to bactericidal serum systems, produced greater enhancement of bactericidal effects than the same amount of their constituent amino acids (Table VIII). This last effect might be attributable to the cells' greater capability to take up dipeptides in comparison to amino acids(7).

Discussion. The foregoing data support the

TABLE VI. Effects of Pre-exposure to Basic Amino Acids, plus or minus Broth, upon Subsequent Serum Susceptibility of *Shigella dysenteriae*, S_1 or S_3 Organisms.

	% survivors after 60′ exposure	
Pretreatment with:	S_1	S_3
dl-lysine (10 mg/ml)	46	33
-arginine ”	40	33
-lysine in tryptose broth	91	88
-arginine *idem*	82	75
Serum control	32	29
Serum dil.:	1:2.5	1:10
Inoc. size:	3410/ml	3010/ml

conclusion that the extent of bacterial lysis by serum is greatly dependent upon metabolic activity of bacteria at time of, or very shortly after, serum damage to wall components. This relationship appears similar to the well-established dependence of penicillin lysis upon metabolic activity(6), and may possibly be a reflection of the need for growth and/or cell division to render partially damaged walls lethal for the affected bacterium. However, involvement of more complex interactions between metabolism and serum lysis cannot be ruled out. The present finding that inhibition of protein synthesis by chloramphenicol interferes with bactericidal serum effects, at first appears to be in contrast to prior data (3) on the additive killing effects of serum and chloramphenicol observed with *Salmonella*. However, earlier data were obtained not only with entirely different bacteria but apparently involved the use of bactericidal antibiotic concentrations, whereas only bacteriostatic concentrations have been employed here. One possible explanation for the observed protective effects of basic amino acids could be based on the assumption that these compounds may be able to combine with, and to protect, bacterial surface sites which act as receptors for bactericidins. The proper conditions for such combinations may exist only in environments with approximately neutral or slightly basic pH. Whereas such combinations appear easily dissociable after exposure in a nutrient-free environment, their irreversibility after exposure in the presence of nutrients might reflect a need for growth or active metabolism to permit firm attachment or partial penetration of basic amino acids into the bacterial wall. Studies are under way to elucidate the nature of these effects and to test the capability of specific carbohydrates and amino acids to modify bactericidal serum effects *in vivo*.

TABLE VII. Effects of Addition of Single and Mixed Amino Acids on Serum Susceptibility of *Shigella dysenteriae*, S_1 and S_3.

Amino acids added	Conc., mg/ml	% survivors after 60′ exposure S_1	S_3
Single additions			
dl-glutamic acid	1	39	36
-aspartic "	1	44	41
-alanine	1	53	46
-lysine	10	89	87
Mixed additions			
dl-glutamic acid	.33	13	23
-aspartic "	"		
-alanine	"		
-glutamic acid	.5	83	85
-aspartic "	.5		
-lysine	10		
None (serum control)		70	58
	Serum dil.:	1:5	1:20
	Inoc. size:	3140/ml	2650/ml

TABLE VIII. Effects of Dipeptides on Serum Susceptibility of *Shigella dysenteriae*, S_1 and S_3.

Compound added	Conc., mg/ml	% survivors after 60′ exposure S_1	S_3
l-lysyl-glycine	1	12	16
-lysine + glycine	.5 of each	37	35
Glycyl-dl-phenyl-alanine	1	14	19
Glycine + dl-phenyl-alanine	.5 of each	33	37
Glycyl-l-glutamic acid	1	13	18
Glycine + l-glutamic acid	.5 of each	30	34
dl-alanyl-dl-alanine	1	17	22
-alanine	1	29	33
None (serum control)		41	47
	Serum dil.:	1:3	1:15
	Inoc. size:	3710/ml	2920/ml

Summary. Studies with *Shigella dysenteriae* and *Escherichia coli* strains revealed enhancement of bacterial susceptibility to normal human serum *in vitro* in the presence of low concentrations (0.1%) of broth, of glucose, or of certain amino acids known to play a prominent role in bacteria's metabolism. Dipeptides containing these amino acids were more effective than single amino acids. In contrast, metabolic inhibitors, including 5-methyltryptophan, fluorophenylalanine, and non-bactericidal concentrations of chloramphenicol, decreased bacterial susceptibility to serum. Such decrease also was obtained in the presence of, or following exposure to, relatively high concentrations (0.5%) of so-called basic amino acids. The latter effects were reversible by washing when bacteria had been exposed in the absence of nutrients, but they were irreversible following exposure in the presence of nutrients.

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Received July 10, 1959. P.S.E.B.M., 1959, v102.

Influence of Nitrogen Source on Growth of *Streptococcus Bovis*.* (25292)

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Niven *et al.*(1) showed that a number of strains of *Streptococcus bovis* have no absolute requirement for any single amino acid, and that most strains studied could grow in a medium containing arginine as the only source of amino nitrogen. Our previous work has shown growth dependence of 2 strains of *S. bovis* on CO_2 when arginine served as the nitrogen source(2), and demonstrated certain interactions between CO_2 and various individual amino acids during growth in this medium(3). Ability of the organism to grow well in media containing arginine as sole source of amino nitrogen suggested that other compounds might similarly serve in this capacity. This report describes ability of various individual amino acids and other nitrogenous compounds to support growth of 3 strains of *S. bovis* in the presence of added CO_2.

Materials and methods. The cultures were *Streptococcus bovis* ATCC 9809 and 2 cultures from Cornell University collection designated as P-10 and P-2. Maintenance of cultures and preparation of inocula have been described(2). *Basal medium* consisted of glucose, K_2HPO_4, sodium thioglycollate, adenine, guanine, uracil, a vitamin mixture, and salts mixture in the same proportions described by Prescott and Stutts(2). Various nitrogen sources were added for each experiment, usually at levels of 0.05 or 0.1 m mole/6 ml culture, although graded levels of these compounds were frequently tested. Also, some experiments compared responses to different amino acids supplied at the same level of total nitrogen. Filter sterilized $NaHCO_3$ was added to autoclaved medium at 0.05 or 0.2 m mole/6 ml culture to supply the CO_2 requirement (2,3). *Procedures for growth tests* were identical to those used in earlier experiments(2). Growth was determined turbidimetrically, either at a fixed time, or more commonly, at different times during the growth phase. Incubation was usually terminated at 72 hours or less, and was never carried beyond 96 hours.

Results. Ability of compounds to serve as sole nitrogen source.† Thirty-seven amino acids and related organic nitrogenous compounds were tested for ability to serve as sole source of amino nitrogen for 3 strains of *S. bovis*. A list of compounds tested and their effects on 2 strains are shown in Table I. These data represent average responses, and evaluations shown are based on the following factors: (a) total growth, (b) rate of growth, and (c) consistency of growth response from one experiment to another. Results obtained with strain P-2 are not shown, since this culture did not show consistent heavy growth with any of the individual compounds listed in Table I, although it grew well with a complete amino acid mixture, as did the other 2 strains.

Relatively few amino acids tested supported

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* Supported in part, by ONR Contract and by grant from Robert A. Welch Fn.

† Exclusive of vitamins, purines, and pyrimidines. Purines and pyrimidines are not essential for growth under conditions employed(1), but were routinely included in the basal medium to insure more rapid and regular growth.

TABLE I. Ability of Individual Compounds to Serve as Primary Nitrogen Source for Growth of 2 Strains of *S. bovis*.*†

Addition to basal medium	Strain P-10	 9809
DL-α-Amino-*n*-butyric acid	+	—
L-Arginine • HCl	++++	++++
DL-Aspartic acid	+	+
DL-Citrulline	+++	+++
L-Cysteine • HCl	+	+
α, ε Diaminopimelic acid	++	±
D-Glucosamine • HCl‡	+++	++
L-Glutamic acid	±	±
L-Glutamine§	++++	+++
Glutathione (reduced)	±	±
Glycocyamine	±	±
L-Histidine • HCl	+++	++
L-Lysine • HCl	±	±
DL-Methionine	±	±
L-Ornithine • HCl	++	+
DL-Phenylalanine	+	+
L-Proline	±	±
DL-Serine	+++	+++

* Other compounds tested which failed to support growth of either strain were *DL*-Alanine, *DL*-α-Aminoadipic acid, γ-Amino-*n*-butyric acid, α-Aminoisobutyric acid, *DL*-α-Amino-*n*-caproic acid, *DL*-α-Aminophenylacetic acid, *DL*-α-Amino-*n*-valeric acid, *L*-Canavanine • HCl, Glycine, Guanidine, *L*-Homoarginine‖, Hydroxy-*L*-proline, *DL*-Isoleucine, *L*-Leucine, *DL*-Norleucine, *DL*-Norvaline, *DL*-Threonine, *L*-Tyrosine, and *DL*-Valine.

† Some compounds showed variation from one experiment to another in ability to support growth. Serine, for example, produced moderate to good growth in one experiment, yet little or none in the next, thus suggesting existence of some variation in cultural conditions. Careful attention to level of $NaHCO_3$ supplied was frequently beneficial, but did not completely eliminate the inconsistencies. These erratic growth responses were not eliminated by changing the density of inoculum, varying autoclaving time, or by filter sterilizing the nitrogen source.

‡ Effective when filter sterilized and added to autoclaved medium.

§ Effective either filter sterilized or autoclaved with medium.

‖ Kindly supplied by Dr. James B. Walker.

vigorous growth of *S. bovis,* indicating a definite selectivity with respect to nitrogen sources. Of amino acids tested, arginine was, by above criteria, the best nitrogen source for both strains P-10 and 9809. Superiority of arginine was not simply a result of its high nitrogen content, since it supported better growth than other compounds when compared at equal levels of nitrogen. Nutritional selectivity of these organisms is emphasized by the observation that 2 closely related structural analogues of arginine, canavanine and homoarginine, did not support rapid or heavy growth. On the other hand, neither of these compounds inhibited growth in medium containing arginine(3). Several amino acids failed to produce even traces of growth in repeated experiments (Table I); some of these compounds were identical to those which retard growth of this organism in the arginine medium(3).

The possibility existed that the organism might utilize any amino acid as a primary nitrogen source once growth had been initiated. A complete amino acid mixture(4) was therefore added to the basal medium in amounts just sufficient to initiate growth, and some 20 amino acids were tested individually for ability to support growth under these conditions. These low levels of complete amino acid mixture did not markedly increase ability of individual amino acids to serve as nitrogen source.

Unlike most organic nitrogenous compounds, simple ammonium salts produced excellent growth which frequently equalled or exceeded that obtained with arginine. Furthermore, strain P-2, which did not grow consistently well on any single amino acid tested, showed heavy growth in basal medium containing ammonium ions. These results provide an additional example of simple nutritional requirements of *S. bovis,* and also emphasize a rather high degree of nitrogen source selectivity, since most preformed amino acids did not support heavy growth. Fig. 1 compares growth obtained when arginine, ammonium acetate, and a complete amino acid mixture (4) served as nitrogen sources. Although the lag phase was sometimes longer with the ammonium medium, total growth was frequently greater than in the arginine medium. Removal of CO_2 from the ammonium medium by the method of Lyman *et al.*(5) resulted in growth failure as it did in the arginine medium(2).

That ammonium ions were capable of serving as a nitrogen source suggested that the effectiveness of arginine might be due in part to its degradation to ammonia, perhaps by action of arginine desimidase, which converts arginine to citrulline and ammonia in *Streptococcus faecalis*(6,7). This possibility appeared especially likely, since citrulline plus ammonium acetate produced rapid, heavy

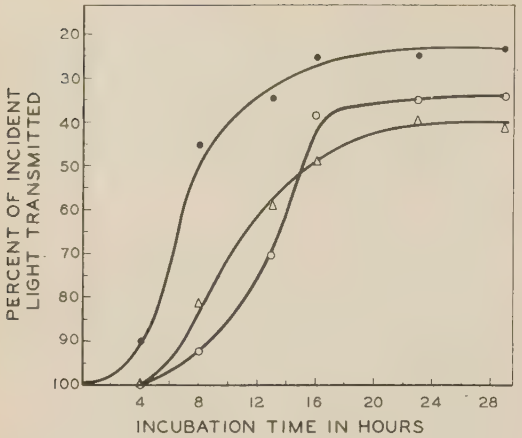

FIG. 1. Growth curves for *Streptococcus bovis* strain P-10, with different nitrogen sources. ●—●, Complete amino acid mixture (30 mg/tube). △—△, *L*-Arginine · HCl (10.5 mg/tube). ○—○: Ammonium acetate (3.85 mg/tube).

growth of all 3 strains. Ability of resting *S. bovis* cells to degrade arginine was therefore tested by incubating suspensions of washed cells with arginine. Neither appearance of ammonia nor destruction of arginine (measured colorimetrically(6)) was observed under conditions employed, nor did addition of glucose or of ATP plus a mineral mixture to resting cells produce detectable destruction of arginine. Experiments with cell free extracts also failed to show a perceptible reduction of arginine. Failure to detect arginine destruction by resting cells does not mean, however, that such reactions do not occur in actively growing cultures.

Ammonium acetate frequently produced more rapid growth than equivalent amounts of either ammonium sulfate or ammonium chloride, and growth failures sometimes occurred with the latter 2 compounds. This suggested either that sulfate and chloride were toxic, or that acetate was stimulatory. The latter was shown to be the case, since addition of 20 mg of sodium sulfate/6 ml culture failed to reduce growth in the medium containing ammonium acetate, and a marked increase in growth response occurred when medium containing ammonium sulfate was supplemented with 10 mg of sodium acetate/tube. Also, cells of strain P-10 growing in ammonium sulfate medium rapidly incorporated C^{14} labeled acetate. *Effects of amino acids on growth in ammonium medium.* Individual addition of certain amino acids to media containing arginine results in either retardation or stimulation of growth of *S. bovis*(3,8). A similar effect was observed when such additions were made to a medium containing ammonium acetate as the nitrogen source. Amino acids which retarded growth in the arginine medium also retarded growth in the ammonium medium. Citrulline and glutamic acid were extremely effective in stimulating growth in the ammonium medium by reduction of lag phase.

It is somewhat curious that these strains of *S. bovis* exert a decided selectivity toward amino acids which they utilize for growth, yet grow luxuriantly in media containing ammonium salts as the primary nitrogen source. This suggests the possibility that while the enzymatic make-up exists for synthesizing amino acids *de novo* from simple precursors, pathways are not available for certain interconversions among amino acids or for degradation of some amino acids to simpler compounds from which cellular amino acids may be synthesized.

Summary. Of 37 organic nitrogeneous compounds tested, arginine was the most effective as a primary nitrogen source for 2 strains of *Streptococcus bovis*. Other compounds supporting growth were glutamine, citrulline, serine, histidine, and glucosamine. The majority of compounds tested, including most amino acids, failed to support growth. Another strain of *S. bovis* did not grow well on any one organic compound tested as a primary nitrogen source. Simple ammonium compounds, however, were extremely effective in serving as nitrogen source for all 3 strains.[‡] Growth in media containing ammonium salts was stimulated by acetate, and C^{14} labeled acetate was rapidly incorporated. No evidence was found for a mechanism involving degradation of arginine by resting cells or cell free extracts.

Technical assistance of Ann Stipp and Bettye Jones is gratefully acknowledged.

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[‡] *Added in proof.* After this paper was submitted for publication, Wolin, *et al.* reported utilization of ammonium salts as sole nitrogen source by other strains of *S. bovis*(9).

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Received July 20, 1959. P.S.E.B.M., 1959, v102.

Effect of Adrenergic Blocking Agents on Fatty Acid Mobilization during Fasting.* (25293)

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Recent studies by Dole and by Gordon and their collaborators(1,2) indicate that a major portion of lipide mobilized from peripheral depots is transported in the form of non-esterified fatty acids (NEFA). It has been demonstrated that concentration of plasma NEFA increases during fasting and that venous blood draining areas rich in adipose tissue contains higher concentrations of NEFA than the blood supplying these areas(2). The additional observation that adipose tissue from fasting rats releases more NEFA *in vitro* than identically treated tissue from fed rats (3) leaves little doubt that the increase in plasma NEFA seen during fasting reflects lipide mobilization. A large body of evidence has been adduced to support the view that fat mobilization from adipose tissue during fasting is a consequence of increased sympathetic discharge(4). Although epinephrine and norepinephrine have been shown to be potent mobilizers of NEFA(2,5), we have shown (to be published) that the adrenal medulla does not play a major role in NEFA mobilization of fasting since adrenalectomized rats maintained with adrenal cortical replacement therapy respond to fasting in the normal fashion with an increase in plasma NEFA. The present paper reports experiments designed to assess the effects of sympathetic blockade at the ganglionic and postganglionic levels on NEFA mobilization during fasting.

Materials and methods. Female rats (Holtzman) weighing approximately 200 g were used in this study. *Blockade of epinephrine induced NEFA mobilization*: Epinephrine was administered subcutaneously as an oil suspension (1 mg in 0.5 ml peanut oil) to 2 groups of rats. One group received in addition 0.5 mg of ergotamine tartrate in a separate subcutaneous injection. A third group received 0.5 ml of the peanut oil vehicle subcutaneously. The animals were deprived of food at time of injection and were killed 3 hours later. *Blockade of NEFA mobilization during fasting*: Ergotamine tartrate (1.5 mg/rat, total dose) and hexamethonium bromide (15 mg of ion per rat, total dose) were administered in 3 equal subcutaneous injections 0, 4, and 8 hours following removal of food. Dibenzyline was injected under light ether anesthesia as a single dose of 0.5 mg into the tail vein of rats at the beginning of the fasting period. In all cases, control animals were injected with an equal volume of saline by the same route and at the same times. The animals were sacrificed 12 hours after initiation of the fast. At the end of the fasting interval, the animals were anesthetized with ether and blood for NEFA analysis was drawn into a heparinized syringe from the abdominal aorta. NEFA analyses were performed in duplicate by the method of Dole(6) on one ml aliquots of plasma.

Results. The NEFA mobilizing action of epinephrine is completely blocked by 0.5 mg of ergotamine (Table I). None of the au-

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* Supported by grant from Am. Cancer Soc.
† U.S.P.H.S. Predoctoral Fellow, Nat. Cancer Inst.

TABLE I. Effect of Ergotamine on NEFA Mobilizing Action of Epinephrine. Six animals/series.

Treatment	Plasma NEFA, μEq/ml	P
Control	.529 ± .055*	
Epinephrine	.724 ± .047	<.05
” + ergotamine	.572 ± .016	<.45

* Mean ± S.E.

tonomic blocking agents used had any significant blocking effect on NEFA mobilization during fasting (Table II).

Discussion. These experiments suggest that sympathetic discharge does not initiate NEFA mobilization in response to fasting, since pharmacological blockade at either the effector site (ergotamine and Dibenzyline) or the ganglionic level (hexamethonium) failed to prevent NEFA mobilization. The fact that a single injection of ergotamine completely blocks the NEFA mobilizing action of a large dose of epinephrine indicates that adrenergic mobilization of NEFA is susceptible to pharmacological blockade. Wool *et al.*(7) were able to block the accumulation of liver lipide in the ethionine treated rat with 0.5 mg of ergotamine or adrenal demedullation suggesting that the fat mobilizing action of endogenous epinephrine or norepinephrine is also subject to pharmacological blockade. From these results it would appear that ergotamine was used in adequate dosage to effect at least partial blockade of epinephrine and norepinephrine. Judging the adequacy of the dosage of the Dibenzyline is more complex. Harvey *et al.*(8) reported that 15 mg/kg of Dibenzyline brought about a 70% suppression of the hyperglycemic response to an intravenous dose of 1 μg/kg of epinephrine in rabbits. Nickerson *et al.*(9) found that 3 mg/kg of Dibenzyline could block the effects of 5 μg/kg of epinephrine or norepinephrine on the blood pressure of the cat. There is thus a wide disparity in dose of Dibenzyline necessary to block the different parameters of adrenergic activity and perhaps species differences as well. This is further complicated by our findings (unpublished) that large doses of Dibenzyline (15-25 mg/kg) cause an increase rather than a decrease in NEFA concentration in rats during fasting. The dose of hexamethonium used in these experiments is in excess of the dose (2-5 mg/kg) found to exaggerate the hypoglycemic effects of insulin and reported to produce "effective pharmacological sympathectomy" in the dog(10). Nevertheless, any demonstration that depends solely upon pharmacological blockade is subject to criticism on the grounds that effective blockade may not have been achieved.

TABLE II. Effects of Autonomic Blockade on Plasma NEFA at End of a 12-Hour Fast.

Treatment	No. of animals	Plasma NEFA, μEq/ml	P
Non-fasted	27	.286 ± .027*	
Saline†	6	.654 ± .056	
Ergotamine†	6	.604 ± .045	<.6
Hexamethonium†	6	.585 ± .045	<.3
Saline	5	.508 ± .041	
Dibenzyline‡	6	.664 ± .058	<.1
Saline	5	.632 ± .029	
Ergotamine‡	6	.564 ± .052	<.3

* Mean ± S.E.

† Administered in 3 doses at 0, 4, and 8 hr following removal of food.

‡ Administered as single dose of 0.5 mg at time of food removal.

Our findings that the NEFA mobilization of fasting cannot be suppressed by autonomic blockade agree with the recent report by Levy and Ramey(11) that they could not prevent loss of fat from the epididymal fat body of the fasting rat using autonomic blocking drugs. However, the apparent absence of effect of the sympathetic nervous system on NEFA mobilization during fasting is in direct conflict with reports(4,12) that denervated adipose tissue contained more fat than normally innervated contralateral tissue following a fast. From these studies it was inferred that sympathetic nervous stimulation induces fat mobilization. However, Clement and Shaeffer(13) have shown that denervation interferes with the mobilization of lipide induced by injection of a large dose of epinephrine. But, if sympathetic discharge and hence secretion of an epinephrine-like mediator is necessary for mobilization of lipide from adipose tissue, one would expect the response to exogenous epinephrine to be unaffected or perhaps even enhanced by denervation(14). It should be noted that epinephrine and norepinephrine have been shown to mobilize NEFA effectively when added directly to adipose tissue incubated *in vitro* and hence devoid of nervous connections(3,15). Further experimentation will be necessary to resolve these paradoxical findings.

Summary. Repeated doses of ergotamine and hexamethonium had no effect on increase in plasma non-esterified fatty acid (NEFA) concentration which occurs during fasting. Dibenzyline was similarly without effect on this response. These findings suggest that the stimulus to increased NEFA mobilization during fasting may not be mediated by the adrenergic nervous system.

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Received July 27, 1959. P.S.E.B.M., 1959, v102.

Effect of Injection of Rabbit Leucocytes into Neonatal Rabbits on Subsequent Lymph Node Cell Transfer.* (25294)

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If rabbit lymph node cells are incubated with filtrate of trypsin-treated *Shigella paradysenteriae* and transferred to fresh rabbits, agglutinins to *Shigella* appear in sera of recipients in a few days. It has also been shown (1) that if blood leucocytes obtained from prospective donor rabbits are injected into recipients a week before cell transfer, such agglutinins do not appear. Failure of agglutinins to appear indicates that injection of leucocytes before cell transfer has altered the host environment with respect to transferred cells, with the result that these cells become ineffective in production of agglutinins. This suppressive effect on transferred lymph node cells was considered the result of a homograft reaction, as originally described by Medawar (2) in the case of skin grafts. The present study arose from attempts to produce in the lymph node cell transfer system another, essentially opposite, effect of tissue transplantation, that of acquired tolerance. This state was first experimentally induced by Billingham, Brent and Medawar(3) by injection of fetal mice of one inbred strain with tissue cells obtained from another strain of mice; after birth, injected mice showed tolerance to tissues of donor strain by accepting skin homografts from this strain. Similar results were reported by these authors with rabbits injected during fetal life with rabbit spleen cells(4). Later Billingham and Brent(5) established acquired tolerance even in mice injected soon after birth with spleen cells of donor strain. In rabbits these authors reported that intravenous injection of the newborn with homologous thymocytes or spleen cells resulted in induction of weak tolerance, in only a small

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* This study was supported by Research Grant of Nat. Heart Inst., N.I.H., U.S.P.H.S.

TABLE I. Maximum Agglutinin Titers of Recipients of *In Vitro* Incubated Lymph Node Cells; Comparison between Recipients Injected with Donor Leucocytes Near Birth, and Those Not Injected with Donor Leucocytes.

Age of rabbits at time of:			Max agglutinin titers of recipients inj. with donor leucocytes:		
Inj. of donor leucocytes	Cell transfer	No. of cells transferred, $\times 10^6$	Soon after birth and again 1 wk before cell transfer	Soon after birth only	Max agglutinin titers of control, 1-kg, recipients not inj. with donor leucocytes
1 day	80	152	<12, <12, 24		128, 128
1	70	179	<12, <12, <12, 12		128
2	67	"	<12		256
2 hr	64	"	<12		384
10 "	74	206		16	96
2 days	81	204		24	384, 384
8 hr	75	206		<12, 48, 64	128, 192, 384, 512
1 day	60	"		12, 32, 64, 64	192, 384
1	52	172		<12, <12, 32, 48	128, 128, 512, 512
1	36	"		<12, <12, <12, 32, 96	256
1	43	222		<12	

proportion of the animals(6). The present study was begun to determine whether injection of leucocytes of adult rabbits into neonatal rabbits would lead to a state, referable to immunologic tolerance, in which transferred lymph node cells would be better accepted by tissues of recipient, with the result of higher agglutinin titers in sera of these recipients. This was not achieved; on the contrary, evidence was obtained that injection of leucocytes very early in life induced the lymph-node-cell-suppressive reaction.

Materials and methods. Injection of newborn rabbits with donor blood leucocytes. Prospective donors of lymph node cells were bled by cardiac puncture, and the heparinized blood was mixed with one volume of 3% gelatin solution in tall narrow tubes. After one hour at room temperature the layer above the sedimented erythrocytes was removed by suction and leucocytes contained therein were collected by centrifugation. The cell sediment was suspended in a convenient volume of Tyrode's solution containing 0.5% bovine plasma albumin and heparin. Newborn rabbits were injected intravenously with 10 x 10^6 donor leucocytes at an interval after birth of a few hours to 2 days. *Cell transfer.* The popliteal and axillary lymph nodes of donor rabbits were excised, and cells obtained from them were incubated *in vitro* with filtrates of trypsin-treated suspensions of *Shigella paradysenteriae,* as described elsewhere(7). After ½ hour incubation at 37°C, lymph node cells were washed and transferred to X-irradiated recipients (425 r of deep X-ray treatment 24 hours earlier) by intravenous route. Thereafter recipients were bled at regular intervals and their sera tested for agglutinins to *Shigella.*

Results. 1. *Attempts to induce tolerance to donors' lymph node cells by injection of neonatal prospective recipients with blood leucocytes from prospective donors.* Newborn rabbits (2 hours to 2 days after birth) were injected intravenously with leucocytes of prospective donors. About 2 months later the same young rabbits were again injected, intradermally, with leucocytes from these donor rabbits, and one week later the young rabbits were given *Shigella*-antigen-incubated lymph node cells from same donors. Injection of leucocytes prior to cell transfer could be expected to result in suppression of transferred cells, as indicated above, unless injection of leucocytes near birth had made the rabbits tolerant of these donor cells. As shown in first 4 rows of Table I, these recipients either developed no measurable agglutinins to *Shigella,* or a very low level of agglutinins, in contrast to control recipients, which had not been injected with donor leucocytes at any time. Suppression of transferred cells in leucocyte-injected animals indicated that tolerance to donors' tissue antigens had not been induced by neonatal injection of recipients with such cells.

The possibility was considered that injection of leucocytes prior to cell transfer might have constituted too severe a test of tolerance to tissue antigens of donors in young rabbits. Accordingly, in the next series of experiments neonatal rabbits were injected with leucocytes of adult rabbits only near the time of birth, and some time later these young rabbits were given antigen-incubated lymph node cells from adult rabbits. It was thought that if a state of tolerance existed which was too weak to overcome the effects of pre-injection of donor leucocytes prior to cell-transfer, this might be indicated by agglutinin titers higher in these recipients than in control recipients, which were not injected during neonatal life with the donors' leucocytes. However, results of these experiments indicated quite the reverse: maximal agglutinin titers of recipients injected with donors' leucocytes early in life were considerably lower than those of control recipients which had not received such an injection (lower part of Table I). These data not only failed to give evidence of a greater degree of acceptance of transferred cells by tissues of the recipient, as a result of neonatal injection of leucocytes, but indicated a suppressive effect on transferred lymph node cells, presumably due to that injection.

With older recipient animals(1) it was found that maximal suppression of lymph node cells occurred when the interval between injection of leucocytes and transfer of lymph node cells was between 7 and 20 days, and that with increasing length of this interval there was gradual decrease of suppressive effect. In the present experiments the interval between injection of leucocytes and transfer of lymph node cells was 36-81 days. To see whether the lymph-node-cell-suppressive effect could be more clearly demonstrated, the following experiments were performed:

2. *Induction of lymph-node-cell-suppressive effect by neonatal injection of leucocytes.* Litters of new-born rabbits were divided into 2 halves. Rabbits in one half of each litter were injected (at age between 8 hours and 1 day) with blood leucocytes from prospective donors of lymph node cells. At various intervals thereafter lymph node cells obtained from these donor rabbits were incubated *in vitro* with *Shigella*-trypsin filtrate and transferred to all rabbits of the litter. Data are shown in Table II. In all cases the anti-*Shigella* agglutinin titers of recipients which had been injected with leucocytes were markedly lower than those of their control littermates. Even with injection of leucocytes at 8 hours of age, and cell transfer on fourth day of life, suppression of transferred lymph node cells was found. Clearly, injection of donor leucocytes near birth had actively induced a reaction of the homograft type against the transferred lymph node cells.

From another type of experiment involving cell-transfer Sterzl deduced that newborn rabbits can react to antigens of leucocytes of other rabbits. Spleen cells were transferred to 5-day-old recipients, *Brucella suis* antigen alone was injected at various intervals thereafter, and agglutinins were subsequently sought in sera of recipients. From the finding that 4 days was the maximum interval consistent with appearance of agglutinins, it was concluded that spleen cells survived no more than 4 days in these neonatal rabbits(8).

In view of this evidence of immunologic activity of the neonatal rabbit, it is of interest that newborn rabbits have been found not to respond (prior to 14th day of life) with active formation of antibody to antigens of *Brucella suis*(9) or to agglutinogen of *Shigella*.

3. *Transfer of* in vitro-*incubated lymph node cells to neonatal rabbits.* Data of control rabbits of Table II, including litters of 8 and 4 days of age, also showed that young rabbits could develop agglutinins after transfer of *in vitro*-incubated lymph node cells. This was explored further, in age range of 1-11-day-old litters. All neonatal rabbits were X-irradiated 24 hours before cell-transfer, then were given lymph node cells which had been incubated *in vitro* with *Shigella*-trypsin filtrate. In these recipients agglutinins developed by fifth day, generally reached a peak by 7th day, and began to decline between 9th and 12th day after transfer. Peak agglutinin titers are shown in Table III, in comparison with those of 1-kg recipients. Agglutinins appeared in neonatal recipients in a wide

TABLE II. Comparison of Maximum Agglutinin Titers of Littermates after Transfer of *In Vitro* Incubated Lymph Node Cells; Part of Litter Injected with Donor Leucocytes Near Birth, Remainder Not Injected.

Age of rabbits at time of: Inj. of donor leucocytes	Cell transfer	No. of cells transferred, $\times 10^6$	Max agglutinin titers of recipients inj. with donor leucocytes soon after birth	Max agglutinin titers of littermates not inj. with donor leucocytes
1 day	31	222	<12, <12, 32	384, 512, 512, 768, 3072
10 hr	30	"	<12, 64	512, 512, 768, 2084
1 day	8	182	32, 48, 64	1024, 1536, 1536
1 *	8	144	<12, 12, 12	128, 256
8 hr	4	140	16, 16	128, 256
		70	16	

* Donor leucocytes inj. intradermally. All other groups were inj. intrav.

range of titer, often at a relatively high level and occasionally near the threshold of measurement.

In these experiments neonatal recipients were X-irradiated prior to cell-transfer, as were the 1-kg recipients. In internal comparisons within litters, agglutinin titers of irradiated recipients were higher than those of their non-irradiated littermates. In other experiments comparisons were made of transfer to neonatal rabbits of lymph node cells obtained from uninjected donor rabbits and from rabbits which had previously been injected in the foot pad with a heterologous antigen (human gamma globulin) to increase the yield of lymph node cells. No differences were found between geometric mean peak titers of groups of littermates receiving cells from these 2 sources.

Finding of agglutinin titers, especially in a relatively elevated range, in neonatal recipients indicates that these recipients can, to some extent at least, provide a suitable environment for the functions of transferred cells. A full evaluation of the relative effectiveness of lymph node cells obtained from adult donors in 1-kg recipients and in neonatal recipients would require data which are not yet at hand. However, preliminary evaluation can be made by calculations involving the numbers of cells transferred and relative weights of recipients of the 2 groups. Relative effectiveness of cells in the 2 situations was estimated by multiplying for each type of recipient, the geometric mean maximal agglutinin titer, per million cells, by mean body

TABLE III. Transfer of *In Vitro* Incubated Lymph Node Cells to Irradiated Neonatal Recipients (1-11 Days) and to 1-kg Rabbits.

Age of neonatal rabbits at time of cell transfer (days)	Mean wt of rabbits (g)	Lymph node cell transfer to neonatal recipients: No. of cells transferred ($\times 10^6$)	Max agglutinin titers of recipients: Individual peak titers	Max agglutinin titers of recipients: Geometric mean ($\log_2$)	Lymph node cell transfer to 1-kg recipients: No. of cells transferred ($\times 10^6$)	Max agglutinin titers of recipients: Individual peak titers	Max agglutinin titers of recipients: Geometric mean ($\log_2$)
11	150	138	96, 192, 192, 256, 384, 384, 384, 512, 512, 1024	7.5	138	96, 96	6.5
9	130	184	128, 384	7.8	184	384, 1024, 1024	9.5
4	80	87	256, 256, 384, 512, 768, 1024	8.8	194	96, 384	7.5
4	80	115	24, 384, 384, 512, 1024	8.3	156	128, 128, 384	7.3
2	60	43	192, 384, 384, 768	8.5	127	96, 1024	8.3
1	50	187	384, 384, 384, 512, 512, 768	8.8	187	128, 384	7.8
1	50	152	768, 768	9.5	152	64, 256	7.0

weight of recipients (Table III). In 5 of 7 experiments the ratio of effectiveness of cells when transferred to 1-kg recipients, to that found on transfer to neonatal recipients, ranged from 2.2 to 4.9. In the other 2 experiments this ratio was 10 and 24, respectively.

In a study by Dixon and Weigle(10) lymph node cells obtained from donor rabbits injected 2 hours earlier with *Shigella*-trypsin filtrates were transferred subcutaneously or intramuscularly into 5-day-old recipient rabbits. Agglutinins failed to appear in sera of 5-day-old rabbits, although agglutinins were found subsequently in sera of adult control recipients. A possible basis for this difference in findings may be that in our experiments lymph node cells were incubated *in vitro* with the antigen, and were transferred by intracardiac injection, and that neonatal recipients had been irradiated. However, in a more recent study Dixon and Weigle did not observe substantial differences in peak titers of neonatal recipients whether they were irradiated or not, or whether lymph node cells were given by subcutaneous or intracardiac injection (11).

In other studies involving transfer to neonatal rabbits of cells of rabbit lymph or spleen, Sterzl(12) transferred to 5-day old rabbits, by intraperitoneal injection, spleen cells which had been incubated *in vitro* with *S. paratyphi* and found agglutinins subsequently in sera of recipients, in range of titers of 8 to 128. Also, Holub(13) transferred to 2-5-day-old rabbits, by the same route, cells collected from the cisterna chyli or spleens of adult rabbits and incubated *in vitro* with *Brucella suis* or *Salmonella paratyphi B.* Agglutinins appeared in these recipients in range of 2-64.

Summary. 1. Newborn rabbits were injected with leucocytes obtained from adult rabbits which were prospective donors of lymph node cells. When antigen-incubated lymph node cells from these donors were transferred to young rabbits 1-2½ months later, agglutinins failed to appear, or did so in low titer. This suppression of transferred lymph node cells indicated that tissues of newborn rabbits had reacted to antigens of leucocytes of donor animals. 2. Transfer of lymph node cells incubated *in vitro* with *Shigella*-trypsin filtrate to uninjected newborn rabbits (1-11 days of age) resulted in the appearance of anti-*Shigella* agglutinins.

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Received July 31, 1959. P.S.E.B.M., 1959, v102.

Mechanism of Aminonucleoside-Induced Nephrosis in the Rat. I. Metabolism of Tritiated Aminonucleoside.* (25295)

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Structural similarity of adenosine and the aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β - D - ribofuranosyl)-purine, suggests that the mechanism by which the lat-

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* This investigation was supported in part by grant-in-aid from Michigan Kidney Disease Fn.

ter induces nephrosis in the rat may well involve interference with adenosine metabolism at the nucleoside, nucleotide, or nucleic acid level. Thus, for example, incorporation of aminonucleoside into molecular structures closely resembling those of adenosine nucleotides, adenosine-containing cofactors (such as diphosphopyridine nucleotide and coenzyme A), ribonucleic acids, or deoxyribonucleic acids, might result either in fouling of normal metabolic pathways by which such compounds are biosynthesized or in blocking of metabolic processes in which the aminonucleoside-containing structures fail to substitute for their adenosine counterparts. Proof that aminonucleoside is metabolized to any one of the indicated structures would, of course, furnish support for such a mechanism; and in addition serve as a guidepost to subsequent investigation. With this in mind, studies were undertaken of the metabolism of aminonucleoside labeled with tritium by the Wilzbach procedure. The present report focuses attention primarily on the chemical nature of tritium activity in an acid-soluble nucleotide fraction obtained from kidney tissue of rats rendered experimentally nephrotic with the aminonucleoside. In addition, it presents data on distribution of radioactivity in body fluids and urine, as well as in kidney and liver nucleic acids.

Procedures. Tritiated Aminonucleoside. One g of aminonucleoside[†] was exposed to 2.5 curies of tritium at 27°C and 0.4 atmos for 2 weeks. The labeled material was dissolved in water, which was later removed with vacuum at room temperature. This operation was repeated twice. The material was then lyophylized from distilled water 5 times, until percentage of total activity labilized and exchanged with hydrogens of water/lyophylization had reached a constant value. Samples of labeled aminonucleoside, unlabeled aminonucleoside, and a combined sample of labeled and unlabeled material yielded identical R_f values when chromatographed on Whatman No. 1 paper by descending development with the solvent system, n-butanol saturated with water. *Radiochemical purity* was established by cutting the chromatograph of the labeled aminonucleoside into 10 mm segments, extracting each segment with distilled water and determining the ultraviolet absorption and tritium activity of each extract. Radioactivity was found only in the segment from the aminonucleoside spot. The final material had a specific activity of 10.3 μc/ml. To study distribution of tritium activity in the labeled material, the aminonucleoside was hydrolyzed with 0.5 N sulfuric acid, and the 6-dimethylaminopurine separated as the silver salt from the 3-amino-3-deoxyribose moiety. Determination of the tritium activity of each fraction indicated 5.9% of the activity in the 6-dimethylaminopurine and 94.1% in the 3-amino-3-deoxyribose. *Measurements of radioactivity.* Sample vials were prepared by adding in the following order: 1 ml molar Hyamine, 5 ml absolute ethanol, 0.5 ml or less of sample solution, volume of water required to bring total volume of sample and water to 0.5 ml, and 10 ml of a counting solvent solution. The latter was composed of 1 g of 2,5-diphenyloxazole (PPO) and 0.025 g of 1,4-di-2-(5-phenyloxazolyl)-benzene (POPOP) in 500 ml toluene. All samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 314 long enough to give a 99% confidence interval with a 2% error for the counting rate. All data are corrected for quenching effects and background. The overall counting efficiency for water in our system was 2.4%. *Induction of experimental nephrosis.* The procedure for inducing nephrosis in normal adult Sprague-Dawley virgin female rats was essentially the same as that described by Fiegelson, Drake, and Recant(1). Each of 3 rats, maintained in separate metabolism cages and fed Rockland rat checkers *ad lib,* was injected subcutaneously with the tritiated aminonucleoside, 1.5 mg (about 5 μmoles)/100 g body weight/day for 10 days. The material was injected in the form of a 1.5% solution in isotonic saline. Twenty-four hour urine collections were made for each animal and checked daily for development of proteinuria. Trace proteinuria, normally found in most rats, increased progressively after the 5th or 6th day of aminonucleoside administration and was very marked in all animals by the 10th day. Twenty-four hours after final injection of labeled aminonucleoside, the rats were sacrificed by ether anesthesia and exsanguination. At sacrifice considerable ascites was present. Samples of both blood and ascitic fluid were obtained for tritium assay. *Isolation of BaRNA and BaDNA.* Kidney and liver tissues were immediately removed and either frozen on dry ice and stored at –15°C or processed immediately by the procedure of LePage and Heidelberger(2) to isolate the barium salts of the ribonucleotides (BaRNA) and deoxyribonucleic acid (BaDNA). In general, this involves removal of acid-soluble nucleotides by repeated homogenization of the tissues in cold (0-3°C) 4% perchloric acid, separation of the 4% perchloric acid extracts by centrifugation, extraction of the tissue residue to separate the ribonucleotides from deoxyribonucleic acid, and finally precipitation of the ribonucleotides and deoxyribonucleic acid as barium salts. *Study of chemical nature of tritium activity in acid-soluble nucleotide fraction.* Acid-soluble nucleotides in the 4% perchloric acid extract were adsorbed on Norit A and then eluted with 25% ethanol containing 0.5% ammonia. Following adjustment of the pH to 1.5, the eluate was extracted with ice-cold ether to remove ultraviolet absorbing non-nucleotide material, and then aerated to remove excess ether from the aqueous solution. The pH of the solution was adjusted to 7.5 and the solution chromatographed on a Dowex 1-X4 column, according to the procedure of Pontis and Blumson(3). Ten ml fractions were collected and the elution of the nucleotides followed by measuring the absorption at 260 mμ in a Beckman DU Spectrophotometer. Absorbance ratios were determined for 250/260 and 270/260 mμ measurements, and complete ultraviolet spectra run to obtain characterizing wave lengths of maximum and minimum absorption for each peak eluted. Aliquots of each peak were assayed for tritium activity as described above. Final identification of radioactive peaks was accomplished by isolating the nucleotide as the calcium salt, decomposing the latter with ammonium oxalate, and chromatographing an aliquot of each nucleotide solution along with known nucleotide solutions on Whatman No. 1 paper developed with System III(4).

[†] We are especially indebted to Dr. J. M. Ruegsegger of Lederle Medical Research Section, Am. Cyanamid Co. for generous supplies of aminonucleoside and closely related compounds.

TABLE I. Distribution of Tritium Activity* in Body Fluids.

Fluid	Activity
	Counts/min./ml plasma
Protein-free filtrate of plasma (trichloroacetic acid precipitated)	3860
	Counts/min./ml
Plasma water†	2895
Ascitic fluid	4335
Ascitic fluid water	4020
	Counts/min./10 day collection
Urine	1.27×10^6
” water	2.85×10^5

* Total activity administered over 10-day period $= 3.82 \times 10^6$ counts/min.

† Water samples obtained from body fluids by evaporation of samples of fluid in a Petri dish at room temperature and condensation of the water vapor on the dish cover cooled with dry ice.

Results. Table I summarizes a typical distribution of tritium activity in body fluids of one of the rats after the 10-day period of treatment with labeled aminonucleoside. The data indicate that approximately ⅓ of administered activity was excreted in the urine, and that 22.4% of urinary activity was in the form of water. The activity in plasma and ascitic fluid respectively was 75% and 95% due to labeled water. Study of the chemical nature of radioactivity in a protein-free filtrate of urine from this animal, concentrated on a Celite-Norit A column, eluted with 50% aqueous ethanol, and chromatographed on an Amberlite IR-50-Type 2 column developed with 2 N NH_4OH, revealed the presence of only one radioactive ultra-violet absorbing component. Absorbance ratios and wave lengths for maximum and minimum absorption identified this material as aminonucleoside.

Distribution of tritium activity in various fractions obtained during isolation of the barium salts of the ribonucleotides (BaRNA) and deoxyribonucleic acid (BaDNA) follows the same general pattern in both kidney and liver tissue (Table II). A detailed study of the chemical nature of radioactivity in the

TABLE II. Distribution of Tritium Activity in Acid-Soluble Nucleotide and Nucleic Acid Fractions of Kidney and Liver Tissue.

Fraction	Activity Kidney	Liver
	Counts/min./g tissue extracted	
Acid-soluble nucleotide	7280	5884
	Counts/min./mg/barium salt	
BaRNA	18	25
BaDNA	1	3
Tissue residue	None	None

acid-soluble nucleotide fraction obtained from pooled kidney tissue of all 3 rats, revealed that, out of a total of 8 nucleotide peaks separated by column chromatography, only 2 were significantly labeled with tritium. Table III summarizes absorbance data and wave lengths of maximum and minimum absorption for each of these peaks, referred to simply as Peaks 1 and 2. It is readily apparent that absorbance data for these peaks corresponds respectively with similar data for cytidine and adenosine nucleotides. Final identification of Peak 1 as cytidine diphosphate and of Peak 2 as adenosine monophosphate was completed by means of paper chromatography. The calcium salt of cytidine diphosphate had a specific activity of 24 counts/min./mg, and that of the adenosine monophosphate 106 counts/min./mg.

Discussion. The present studies fail to provide any evidence for metabolism of labeled aminonucleoside into molecular structures closely resembling those of adenosine nucleotides or adenosine-containing cofactors such as diphosphopyridine nucleotide. This negative result would seem to eliminate the possibility of aminonucleoside nephrosis being induced by a mechanism involving biosynthesis of counterfeit or fraudulent nucleotides or cofactors which foul or inhibit normal metabolic pathways requiring their adenosine counterparts.

The observation of tritium activity in adenosine and cytidine nucleotides, however, provides positive information concerning metabolism of the aminonucleoside. In this respect, calculations clearly indicate that labeling to the extent observed in either group of nucleotides can not be accounted for in terms of an exchange mechanism involving hydrogens of body water. This is true whether one assumes that all the hydrogens of each nucleotide can be exchanged with those of tritium-labeled body water, or that only the hydrogens attached to the amino group in the 6 position of the purine or pyrimidine moieties and those of the hydroxyl groups of adenosine and cytidine are exchangeable. Thus, for example, water in plasma, which for all practical purposes can be assumed representative of body water, counts 2895 counts/min./ml or 27 counts/min./mg hydrogen. Assuming all the hydrogens of the adenosine nucleotide to be replaceable, an activity of 3400 counts/min./mg body water hydrogen would be required to label the nucleotide to the extent of 106 counts/min./mg calcium salt. Similar calculations for the labeling of the cytidine nucleotide indicate an activity of 811 counts/min./mg body water hydrogen as a requirement. If one assumes only the hydrogens of the hydroxyl and amino groups to be exchangeable, then body water hydrogen of still higher activity is required.

Clearly then, the observed labeling of the nucleotides must be considered indicative of either direct or indirect metabolism of the tritiated aminonucleoside to labeled products subsequently utilized in the biosynthesis of acid-soluble nucleotides. A possible pathway, for example, might involve enzymatic splitting of the aminonucleoside at the glycosidic bond followed by demethylation of the 6-dimethylaminopurine to yield precursor adenine, and oxidative deamination of 3-amino-3-deoxyribose followed by reduction of the ketopentose to yield precursor ribose. Still another pathway—possibly more important in terms of adenosine metabolism and mecha-

TABLE III. Absorption Data for Acid-Soluble Nucleotides at pH 2.0.

Nucleotide	Absorbance Max	Min	Absorbance ratios 250/260	270/260
	mμ	mμ		
Peak #1	278	242	.57	1.50
#2	257	230	.84	.69
Adenosine phosphates	257	230	.84	.71
Cytidine phosphates	280	241	.45	1.71
Aminonucleoside	268	233	.59	1.14

nism of action of aminonucleoside—might involve a reaction sequence in which the aminonucleoside is metabolized directly to adenosine without splitting at the glycosidic bond. Such a pathway, occurring at the nucleoside level of metabolism, would of course involve the same series of reactions suggested for the 6-dimethylaminopurine and 3-amino-3-deoxyribose moieties. Pertinent in connection with such a metabolic pathway are a number of experimentally determined facts which indicate that intactness of the aminonucleoside molecule is a requirement for the induction of experimental nephrosis in the rat. Thus, in our laboratory (unpublished observations) and in that of a number of other investigators (5), several substances closely related to the aminonucleoside have been tested for their nephrotoxic action. 6-Dimethylaminopurine, 3-amino-3-deoxyribose, a combination of these, 6-dimethylamino-9-(β-D-ribofuranosyl)-purine, and 3′-amino-3′-deoxyadenosine all failed to produce nephrosis under conditions similar to those described in the present report. Only the aminonucleoside and the antibiotic, puromycin, from which it was obtained, were active.

While these investigations with tritiated aminonucleoside were in progress, observations were reported from a number of other laboratories which, in conjunction with the present ones, focus attention on the possibility that aminonucleoside nephrosis may be induced by a mechanism in which unaltered aminonucleoside simply inhibits some key step in the normal metabolic sequence of nucleoside or nucleotide metabolism. In this context, observations that 6-dimethylamino-9-β-D-ribofuranosyl-purine and 6-amino-9(3′-amino-3′-deoxyribofuranosyl)-purine inhibit the conversion of 5-amino-4-imidazole carboxamide riboside to the corresponding ribotide(6), and that the aminonucleoside inhibits production of acid-labile phosphate (ATP) by a crude brewer's yeast system in the presence of adenosine and phosphate(7) suggest interference with phosphorylation mechanisms.

Summary. 1. The preparation and metabolism of radiochemically pure aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine, labeled with tritium by the Wilzbach procedure, is described and discussed. Data are presented for the distribution of radioactivity in body fluids and urine as well as in kidney and liver nucleic acids. 2. Study of the chemical nature of tritium activity in an acid-soluble nucleotide fraction, prepared from pooled kidney tissue of rats rendered nephrotic with the tritiated aminonucleoside, indicates that the rat is capable of metabolizing the latter into labeled products utilizable in the biosynthesis of adenosine and cytidine nucleotides. 3. Labeling of these nucleotides, which cannot be accounted for in terms of an exchange mechanism involving the hydrogens of body water, is considered in terms of alternative metabolic pathways.

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Received August 27, 1959. P.S.E.B.M., 1959, v102.

Passive Transfer of Protection Against Lethality of Homologous and Heterologous Endotoxins. (25296)

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The endotoxin-tolerant animal exhibits striking resistance not only to many effects of a variety of endotoxins, but to such insults as hemorrhagic or traumatic shock or infection by Gram-negative pathogens. It is believed that this tolerance is non-immunological and cannot be passively transferred by blood(1). A limited protection against lethality of endotoxins, or the contributing organism, has been described using classical immunological technics to develop high specific antibody titers in donor animals(2,3,4). The success of such procedures has been demonstrated(3, 4) to depend upon sufficiently high antibody titer in the donor to exceed a critical value for the ratio of antibody to magnitude of challenging dose of toxin in the recipient. Further, this protection was restricted to the homologous endotoxin; it did not extend to the toxin of another strain of the same organism. In a largely neglected report Creech, *et al.* (3) were able, also, to show protection by passive transfer in absence of demonstrable antibodies, this requiring relatively large amounts of gamma-globulin taken from mice but not rabbits after a single injection of endotoxin. This protection, too, was restricted to the homologous endotoxin. An apparent protection against the toxin of another strain of the same organism was reported but cannot be considered statistically significant. The present report describes protection of mice against homologous and heterologous endotoxins by passive transfer of serum of endotoxin-tolerant rabbits having low or no antibody titers to the endotoxins. Passive transfer of tolerance to pyrogenicity of endotoxin in the rabbit and studies on the mechanisms involved will be reported separately. In addition, non-specific protection against lethality afforded by serum of normal as well as tolerant rabbits when given intraperitoneally shortly before, but not simultaneously with, the *i.p.* inoculation of endotoxin will be published shortly.

Materials and methods. Endotoxins used were Difco lipopolysaccharides, *S. typhosa* 0 901 and *E. coli* 0127:B8. Rabbits of mixed breed and sex were rendered tolerant by *i.v.* injection of endotoxin for 6 days in doses of 2.5, 2.5, 5, 5, 10, 10 μg and were sacrificed 20 hours after last injection. Typical fever curves with developing tolerance were recorded. Normal rabbits from the same stock provided control serum. Precautions to avoid pyrogen contamination were observed; all glassware was heated at 180°C for 3 hours before use and solutions for dilution were proved non-pyrogenic as were the collected sera. Tests for protection against endotoxin lethality were performed in 15-18 g mice. Schedules for administration of serum are given in the Tables. Normal saline, given by various schedules, did not yield results different from those obtained in untreated mice and such results were combined for control values on lethality of challenging dose. In preliminary experiments *i.p.* LD_{50} for *S. typhosa* endotoxin was 260 μg and for *E. coli* 210 μg. The challenging doses chosen were estimated to represent the LD_{75}: 600 μg for *S. typhosa* and 500 μg for *E. coli*. In retrospect, the former proved a correct estimate but for *E. coli* the lethality was underestimated, the challenging dose representing an LD_{85}. In all experiments untreated or saline-treated groups were challenged along with experimental groups. Deaths were recorded over 72 hours; in most instances deaths occurred within 48 hours. Although serum collected 20 hours after last injection of endotoxin in the donor proved non-pyrogenic and it is known that endotoxin is rapidly cleared from blood of tolerant rabbits(5), it was thought well to include control groups of mice receiving maximum possible endotoxin content of donor blood prior to challenge. This was estimated on the (patently improbable) assumption that total endotoxin given a rabbit over 6 days was present in the serum sample collected on 7th

TABLE I. Protection by *S. typhosa* Endotoxin-Tolerant Rabbit Serum against *S. typhosa* Endotoxin Lethality in Mice.

Exp. and group	No. exp.	Dead/Total	% dead
Controls*	15	97/129	75†
A. .7 or 1 cc serum, i.p., daily, 3 days, challenged on day 4:			
Normal serum	2	13/18	72
Tolerant serum	3	3/19	16‡
Boiled tolerant serum	1	7/8	88
.8 μg endotoxin	1	9/10	90
B. 1 cc serum, i.p., challenged 2-3 hr later:			
Normal serum	2	15/20	75
Tolerant serum	3	1/29	3‡
.8 μg endotoxin	1	8/10	80
C. .5 or 1 cc, i.v., challenged 30 min. later:			
Normal serum	3	20/30	67
Tolerant serum	3	7/30	23‡
.8 μg endotoxin	1	6/10	60

* Untreated or saline inj., then challenged.
† s.e. ± 5.0%.
‡ $p < .001$.

day. The estimate is 0.8 μg/cc. An aliquot of serum was adjusted to pH 5.5 with N HCl, held in boiling water bath 5 minutes, filtered, the coagulum washed with saline and the filtrate (at original volume) adjusted to pH 7 with N NaOH; this relatively protein-free filtrate is referred to as "boiled serum" in the Tables. Antibody content was determined by ring test using undiluted serum and dilutions in saline of the endotoxin as antigen, and by precipitation following incubation. For both endotoxins the undiluted antigen was set at 1 mg/cc and serial dilutions were allowed to react with equal volumes of serum. For statistical analysis the chi squared contingency test was used(6) and significance assumed at $p \leqq 0.01$.

Results. Sera of rabbits rendered tolerant to *S. typhosa* endotoxin yielded maximum titers to homologous endotoxin of 1:16. No demonstrable antibody to heterologous *E. coli* endotoxin was found. Examination of sera of *E. coli*-tolerant rabbits yielded results consistent with those for *S. typhosa*.

The data for lethality in the mouse of *S. typhosa* endotoxin with *S. typhosa*-tolerant rabbit serum are given in Table I. Neither normal rabbit serum nor maximum amount of endotoxin that could possibly be present in the tolerant serum altered the response to the endotoxin challenge. Tolerant serum, by all schedules, effected highly significant protection, which was lost on heating. Protection following intravenous administration rules out donor-serum inactivation of the endotoxin challenge intraperitoneally.

In Table II are given data showing significant protection against challenge with the heterologous *E. coli* endotoxin. Whether the *S. typhosa*-tolerant serum is less potent in protecting against *E. coli* than against *S. typhosa* cannot be determined from these data since a relatively more lethal challenge of *E. coli* endotoxin was used. In limited experiments with *E. coli*-tolerant rabbit serum given *i.p.* to mice 2.5 hours before challenge with *E. coli* endotoxin, saline-injected controls suffered 8/10 deaths, normal serum 8/10, and the tolerant serum 4/10. Using the same serum and schedule and testing with *S. typhosa* endotoxin the results were: saline 12/20, normal serum 7/10, tolerant serum 5/20. It appears then that the difference in degree of protection is based upon the difference in potency of challenge.

Discussion. Low antibody titers to endotoxin would be expected from the weak antigenicity of lipopolysaccharides and from the brief schedule of *i.v.* injections used to develop tolerance. It is clear that the protection described does not depend upon classical immunological mechanisms.

A highly significant degree of protection

TABLE II. Protection by *S. typhosa* Endotoxin-Tolerant Rabbit Serum against *E. coli* Endotoxin Lethality in Mice.

Exp. and group	No. exp.	Dead/Total	% dead
Controls*	8	58/68	85†
B. 1 cc serum, i.p., challenged 2-3 hr later:			
Normal serum	2	18/20	90
Tolerant serum	3	13/29	45‡
C. .5 or 1 cc, i.v., challenged 30 min. later:			
Normal serum	2	16/20	80
Tolerant serum	2	8/20	40‡

* Untreated or saline inj., then challenged.
† s.e. ± 5.1%.
‡ $p < .001$.

against lethality of both homologous and heterologous endotoxins is afforded by passive transfer of serum of a tolerant animal of another species. The success with the *i.v.* route shortly before the *i.p.* challenge rules out direct neutralization of the endotoxin by residual serum, or inactivating factor, in the peritoneum. (It is known(1) that a mixture of endotoxin and antiserum retains the toxicity of the former.) Preliminary experiments indicate that the protective serum of tolerant donors stimulates the reticuloendothelial system of the recipient, as measured by carbon-clearance in the latter. Whether there is equivalent protection against homologous and heterologous challenge cannot be answered unequivocally; the data do not require an assumption of non-equivalence. While it is true that tolerance to, *e.g.*, pyrogenicity by treatment with one endotoxin confers tolerance to pyrogenicity of other endotoxins, whether there is quantitative equivalence of tolerance is far from proved.

Summary. Serum of rabbits rendered tolerant by a brief schedule of endotoxin administration protects mice against the lethality of homologous or heterologous endotoxin and this protective effect is not attributable to antibody content of the serum.

The author is indebted to Dr. Albert S. Gordon for directing his attention to the bacterial endotoxins and for helpful suggestions and criticism. It is a pleasure to acknowledge the skilled technical assistance of Miriam Graff.

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Received September 4, 1959. P.S.E.B.M., 1959, v102.

Effects of Steroid Induced Potassium Depletion on Renal Control of Sodium.* (25297)

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Sodium retention is a common side effect of continuous therapy with corticoids. Another potential effect of prolonged dosage with cortical hormones is potassium depletion. Since potassium deficiency is known to produce significant alterations in renal function (1), we hypothesized that renal management of sodium during steroid treatment might be influenced by this known change in potassium balance. The present experiments compared renal responses of intact dogs to intravenous administration of sodium after they had received large repeated daily doses of nine-alpha-fluorohydrocortisone (FF) with and without potassium supplements.

Materials and method. The experiments were performed on 12 mongrel dogs weighing between 8.1 and 12.7 kg, divided according to weight into 1 control and 2 test groups, each containing 2 males and 2 females. They were housed individually in metabolism cages and offered a daily food ration of 280 g of Gaines meal wetted with 300 ml of distilled water. Their daily dietary intakes of sodium and potassium were 42 and 62 mEq respectively. Dogs in Group A were injected intramuscularly once daily for 3 weeks with FF alcohol (1 mg/kg), suspended in an aqueous vehicle. This regimen usually elicits a negative potassium balance, manifested by a drop in serum potassium and mild muscular weakness(2).

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* The authors thank Dr. Josef Fried of Squibb Inst. for generous supply of nine-alpha-fluorohydrocortisone.

TABLE I. Renal Responses of Dogs to Intravenous Sodium Administration after Repeated Doses of Nine-Alpha-Fluorohydrocortisone (FF) Alone or with Potassium Supplements.*

Glomerular filtration rate	Urinary output	Sodium			
ml/min.		Serum level, meq/l	Filtered†	Excreted	Reabsorbed, %
			meq/min.		
Group A (4 dogs): FF without potassium supplement					
31 ± 5 (<.02)	2.2 ±.6 (ns)	154 ± 1 (<.01)	4.77 ± .84 (<.05)	.16 ±.05 (<.01)	96.7 ±.8 (<.05)
Group B (4 dogs): FF with potassium supplement					
61 ± 9 (ns)	2.9 ±.4 (ns)	153 ± 3 (<.02)	9.33 ±1.24 (ns)	.41 ±.04 (ns)	95.6 ±.3 (<.05)
Group C (4 dogs): Controls					
51 ± 2	2.8 ±.4	143 ± 1	7.29 ± .25	.46 ±.03	93.7 ±.7

Avg values followed by stand. errors. Numbers in parentheses are P values of *t* tests *vs* control data; "ns" denotes not significant.

* Test dogs were given 1 mg FF/kg/day i.m. for 3 wk; supplemented dogs also received 0.15% KCl in their drinking water. Results tabulated here were obtained on the 21st day during the 3rd hour's infusion with 0.85% saline, 5 ml/min.

† No correction for Donnan effect.

We considered dogs in this group to be deficient in potassium, and therefore suitable for testing, only if they exhibited both of these effects. Dogs in Group B received similar doses of FF but, in addition, were given 0.15% KCl in their drinking water. Ferrebee *et al.*(3) found this procedure effective in counteracting signs of steroid induced potassium depletion. Control dogs (Group C) received injections of the aqueous vehicle alone. Effects of different treatments on renal responses to intravenous sodium administration were assessed by clearance technics at the end of the 3-weeks' dosing period. The dogs were anesthetized with intravenous pentobarbital (30 mg/kg), then given an intravenous priming dose of creatinine (60 mg/kg) followed by an infusion (5 ml/min.) of 0.85% saline containing sufficient creatinine (0.06%) for measurement of filtration rate. Renal functions were compared during the 3rd hour of infusion when urinary excretion rates of the 3 groups were roughly comparable. Four 15-minute clearance tests were made during the hour; urinary samples were obtained through an indwelling catheter and arterial blood samples were withdrawn from the femoral artery at the mid-point of each collection period. Sodium and potassium were analyzed by internal standard (Li) flame photometry and creatinine by a modification of the Bonsnes and Taussky technic(4). The significance of the differences between the mean effects produced by the test and control treatments was calculated by the *t* test for small samples(5). A probability of ≤ 0.05 was considered significant.

Results. During the 3-weeks' dosing period the dogs in both test groups showed equal losses in body weight (avg 10%) and developed polydipsia and polyuria. Dogs given FF without supplemental potassium exhibited signs of potassium depletion as evidenced by mild muscular weakness and hypokalemia (avg ± S.E., 2.64 ± 0.15 mEq/l); in contrast, dogs given FF and additional potassium showed no muscular dysfunction and had serum potassium levels (4.85 ± 0.26 mEq/l) comparable to those of the controls (4.86 ± 0.11 mEq/l).

The apparent disparity in potassium economy between the 2 groups of dogs given FF was reflected by differences in their renal responses to intravenous sodium administration (Table I). Although the animals in both groups had significantly higher concentrations of serum sodium and reabsorbed greater percentages of filtered sodium than the controls, urinary excretion of sodium in the deficient dogs was significantly lower than in the controls but in the supplemented animals it was similar to that of the controls. The difference in sodium output between the 2 test groups was caused by differences in their filtered fractions of sodium as determined by glomerular filtration: in the deficient dogs glomerular filtration was reduced but in the supplemented animals this function, although not significantly different from that of the controls, showed a tendency to be augmented.

It is evident from these studies that FF may have different effects on sodium excretion, depending upon degree of potassium depletion. It is also apparent that erroneous conclusions may be drawn from experiments in which urinary excretion of sodium is used as the sole criterion for determining the sodium retaining properties of corticoids. We believe that studies of potassium balance such as those previously reported from our laboratories(2) and of glomerular-tubular functions as reported here are also needed to elucidate the potential long range effects of new adrenal steroids on sodium excretion.

Summary. The loss of potassium attending repeated administration of large doses of nine-alpha-fluorohydrocortisone (FF) to dogs depressed their glomerular filtration rate. In contrast, glomerular function showed a tendency to be augmented in a group of dogs that received supplemental potassium with the steroid. The ability of FF to increase renal tubular reabsorption of sodium was observed under conditions of both potassium depletion and repletion. In the former case sodium retention was enhanced by the reduction in filtration rate but in the latter case no significant retention of sodium occurred, despite its augmented tubular reabsorption.

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Received September 9, 1959. P.S.E.B.M., 1959, v102

Multiplication and Cytopathogenicity of Herpes Simplex Virus in Cultures of Feline Renal Cells.[*] (25298)

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Virus of herpes simplex has been reported to propagate in many tissue culture hosts(1-12); a search of the literature, however, yielded no reference to its growth in feline renal cells. A strain of herpes simplex virus was isolated in our laboratory and its identity confirmed by complement fixation. The purpose of this work was to determine whether the virus would propagate in these cells and to study the cytopathology. This paper describes the cytopathology of herpes simplex virus in cultures of feline renal cells, its behavior in 20 serial passages, and several conditions that influence the type of cell response to this strain of virus.

Materials and methods. Tissue cultures were prepared in 15 × 155 mm tubes and on 11 × 22 mm coverslips in Leighton tubes. Cell cultures of feline renal cells were prepared by the method of Madin(13). Second and third subcultures of human kidney were prepared by trypsin and versene digestion. The nutrient medium was 0.5% lactalbumin in modified Hank's salt solution containing 0.01 M tris (hydroxymethyl aminomethane), adjusted to pH 7.4 and 20% human serum. Prior to inoculation the cultures were rinsed 3 times with Hank's salt solution. The fluid was replaced with 0.5% lactalbumin hydrolysate containing 5% lamb serum. Human amnion cells (FL)(14) were grown in Eagle's medium containing 20% human serum. At the time of inoculation, maintenance medium, consisting of Eagle's basal medium plus 3% horse serum, was placed on the cultures. Streptomycin (0.5 mg/ml) and penicillin (500 u/ml) were incorporated in all media.

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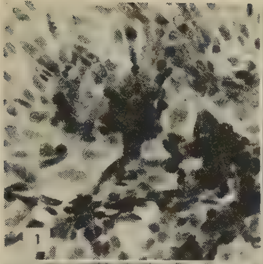
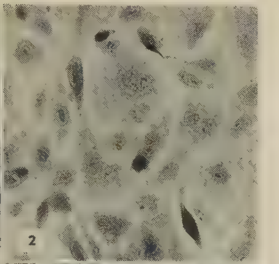
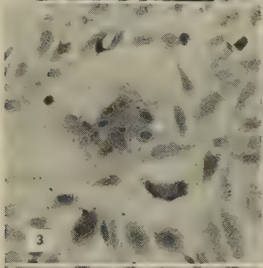
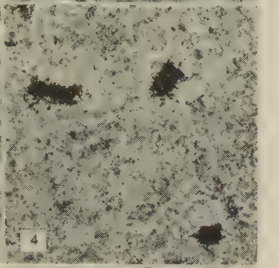
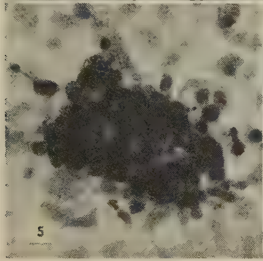
[*] The author acknowledges the work of Yaye F. Herman, Walter Reed Army Inst. of Research, Washington, D.C., in identifying the virus, and the technical assistance of Edward W. Despeaux, Armed Forces Inst. of Pathology, Washington, D.C.











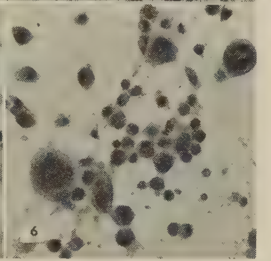


FIG. 1. Proliferative type of lesion in cultures of feline renal cells infected with herpes virus. H & E ×93, AFIP-MIS 58-13112.

FIG. 2. Different stages of nuclear alteration induced by herpes virus in cultures of feline renal cells. H & E ×125, AFIP-MIS 59-2930.

FIG. 3. Multinucleated cell with inclusion bodies in culture of feline renal cells. H & E ×100, AFIP-MIS 59-2931.

FIG. 4. Proliferative type lesion in FL cells infected with herpes virus. H & E ×25, AFIP-MIS 59-2928.

FIG. 5. Same culture as Fig. 4, demonstrating intranuclear inclusion bodies. H & E ×125, AFIP-MIS 59-2927.

FIG. 6. Multinucleated cells in human kidney cell culture infected with herpes. H & E ×100, AFIP-MIS 59-2932.

Titrations of this virus were made in tissue culture by inoculation of 0.1 ml of decimal dilutions into each of 4 tubes. The 50% cytopathogenic end point ($TCID_{50}$) was calculated by the method of Reed and Muench (15). Virus titrations were also made in 21-day-old Swiss albino mice by intracranial inoculation of 0.03 ml of 10-fold dilutions into groups of 6 mice. The mice were observed daily for 3 weeks, and the ID_{50} was calculated by the method of Reed and Muench. The original virus was isolated from a herpetic lesion in primary cultures of human amnion. After one intracerebral passage in mice, the virus possessed an ID_{50} for mice of $< 10^{-1}/0.03$ ml as a 20% brain suspension. The initial inoculum for the feline renal cultures was 0.1 ml of the mouse brain suspension. Subsequent passages were made by transferring 0.1 ml of the undiluted tissue fluid into each of 2 feline renal cell cultures. The fluids from the 2 tubes were pooled and stored at $-60°C$. Coverslip preparations for studying the cytologic changes were stained with hematoxylin and eosin after fixation in Bouin's fluid.

Results. Cytopathogenic changes (CPC) of the proliferative type (Fig. 1) were first observed in cultures of feline renal cells 6 days following the initial inoculation of the mouse brain suspension. The fluids were pooled and passed on the 8th day. In the 2nd feline kidney passage, cell degeneration was present on the 2nd day and the fluid was passed on the 4th day. In all subsequent passages, CPC was observed on the 1st and 2nd days, and transfers were made on either the 2nd or 3rd day.

Results of virus titrations in both mice and cultures of feline renal cells of 10th, 15th, and 20th tissue culture passages are given in Table I.

With an inoculum of 100 $TCID_{50}$ of virus, lesions were first seen in the unstained preparations at 39 hours. These lesions consisted of small foci of rounded refractile cells, which appeared to pile. These areas increased in size, and between 46 and 63 hours new or secondary foci appeared in culture. At this time giant cells were present, and spaces developed in the cell layer. The degeneration generally becomes more diffuse, and eventually the cells are released from the glass surface.

TABLE I. Comparative Titrations of Herpes Simplex Virus Propagated in Cultures of Feline Renal Cells.

No. of virus passage	Virus titer	
	Mice	Feline renal cells
10	2.5 *	5.75†
15	3.83	5.66
20	3.86	5.23

* Log LD_{50}. Intracer. inoculation of 0.03 ml.
† Log $TCID_{50}/0.1$ ml of inoculum.

In stained preparations small foci involving a few cells exhibiting nuclear alterations are evident as early as 22 hours. The early lesion consists of cells with a slightly granular nucleus in which the nucleoli are small or inapparent. In some cells the nucleus is enlarged and the nuclear space is filled with a homogeneous, light-blue material containing numerous small, dark bodies. As the lesion progresses, these basophilic bodies marginate. The nuclear material condenses, and by 39 hours a spherical to oval inclusion is formed within the shrunken but intact nuclear membrane. The inclusion is surrounded by a clear space or halo. In some cells the nuclear material forms small aggregates interlaced with chromatin, which appears as a fine reticulated network. A number of cells exhibiting various stages of nuclear alteration are illustrated in Fig. 2.

At 22 hours post inoculation a few small multinucleated cells containing 2 or 3 nuclei are present. Although some vacuolation occurs in the cytoplasm at this time, it is not considered to be a specific manifestation of herpes infection. Terminally, the cytoplasm clumps around the nucleus and is granular and eosinophilic. When this occurs, the cells may appear round or elongated, with or without protoplasmic processes.

A typical giant cell is composed of an aggregate (syncytium) of cells that have lost their cytoplasmic identity. In the early stage of syncytium formation, the cytoplasm becomes pale and somewhat vacuolated. Most of the nuclei in these giant cells contain inclusions (Fig. 3). Giant cells are most numerous 86 hours after infection.

This study shows that primary cultures of feline renal cells support the multiplication of herpes simplex virus. The 20th tissue culture passage represents a 1×10^{-20} cumulative dilution of the original virus inoculum. Replicate titrations in cultures of feline renal cells show a $TCID_{50}$ of $10^{-5.23}$ to $10^{-5.75}/0.1$ ml. There was a considerable increase in the LD_{50} of the tissue culture adapted virus when titrated into mice (Table I). Identification of the material from the 10th and 20th virus passages was reaffirmed with known herpes immune serum, utilizing the complement fixation and neutralization tests. In addition the virus maintained its infectivity for the embryonated egg. Characteristic intranuclear inclusion bodies were demonstrated in both the chorioallantoic membrane of the embryonated egg and in the infected mouse brain.

Discussion. Virus of herpes simplex has been reported to propagate and elicit a cytopathogenic change in a variety of tissue culture hosts. A review of the literature reveals that the cytopathic changes resulting from herpes infection are primarily of 3 types. These types, as described by Gray, *et al.*(16), are the (1) proliferative, (2) nonproliferative, and (3) giant cell types. In this study we produced all 3 types of CPC with one isolate of virus.

In the human amnion cultures in which the virus was originally isolated, the cytopathic change was of the nonproliferative type. Distinct foci of the proliferative type were seen in cultures of feline renal cells after inoculation with a large amount of virus, whereas a small quantity of virus produced both the proliferative and giant cell types of CPC in equal proportions. The lesion was of the proliferative type in the FL cultures regardless of amount of inoculum (Fig. 4 and 5). The giant cell type was the predominant cytopathic change in human kidney cultures when a large inoculum was given (Fig. 6).

These results indicate that the type of lesion produced is not dependent entirely on strain of virus used but is also influenced by type of cell culture and amount of the inoculum, as shown above. No attempt to clone the virus was made, and it is possible that a mixture of cytopathogenic types of virus may exist. Although the different cytopathogenic changes noted on the various host cells do not rule out this possibility, this study emphasizes the value of using diverse cell systems.

We rarely observed well-developed intranuclear inclusions in our passages of the strain of herpes in human kidney cells. In this respect our results differ from those of Doane *et al.*(5), who report that typical herpetic inclusions were common in human kidney with their strain of virus.

Macroscopic plaques were produced with

our strain of virus in cultures of feline renal cells in the presence of specific antibody. The presence of antibody did not alter the type of lesion when a large inoculum was used to infect the cultures. The microscopic plaques produced in the FL cells were similar in appearance to those described in HeLa cells by Farnham(17).

Summary. The virus of herpes simplex was passed serially 20 times in cultures of feline renal cells. Multiplication of the virus was demonstrated when infectivity titers were measured in mice and in tissue culture. The adapted virus induced cytopathogenic changes in cell cultures of feline kidney, primary human amnion, human kidney, and FL cells. Several conditions which appear to influence the type of cytopathogenic change in cell cultures are discussed.

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Received September 10, 1959. P.S.E.B.M., 1959, v102.

Movement of Radiostrontium Through Intestinal Tract of Fed or Fasted Rats.* (25299)

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During a study of radiostrontium absorption in rats, a simple method was developed for determining its progress through the intestinal tract. The method should prove equally of value in studies using other radioactive substances. It is particularly useful for estimating the amount of radioisotope available for absorption in different segments of the tract at various times after oral administration.

Methods. Adult female rats of Wistar strain were given 3 μc of $Sr^{89}Cl_2$ in 0.75 ml water by stomach tube. One group had been fasted during 24 hours prior to experiment, while the other group was fed *ad lib.* Both groups were subsequently given water *ad lib.*, but no food. They were killed in groups of 4 at ½, 1, 2, 4, 6, and 24 hours, and the intestinal tracts removed and laid out in a straight line. The radioactive material present in successive 1 cm lengths of the gut was determined using a collimated Geiger counting tube, a counting ratemeter, and graphic recorder. The gut can be counted continuously by placing it on moving paper of a kymograph with extender, fixed so that the paper is horizontal.

Results. Fig. 1 shows distribution of radiostrontium at different time intervals in representative animals from the fasted group. Fig.

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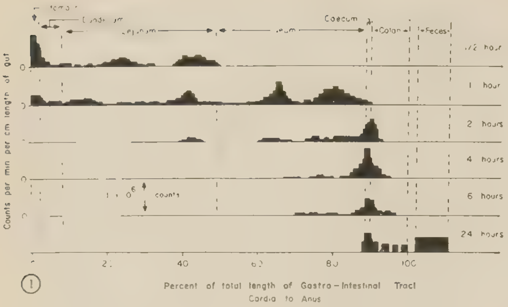
* Supported in part by extramural grant from Defence Research Board, Canada.



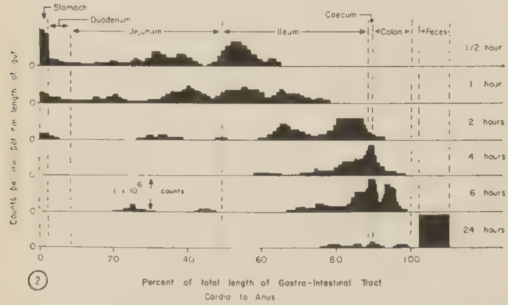


FIG. 1. Location of radiostrontium in intestine of fasted rats.

FIG. 2. Location of radiostrontium in intestine of rats previously fed *ad lib.*

2 shows similar data for the fed group. It is apparent that previous feeding increased the emptying of stomach and movement of the dose through duodenum and jejunum, but resulted in longer retention in the ileum. In the fed group, emptying of colon also occurred earlier. In all cases, distribution of the isotope was not uniform, but was concentrated in separated areas.

The method described using a radio-tracer as indicator of its progress in the gut has advantages over the bead methods of Elliott(1), Alvarez(2) and other use of inert indicators because there is no lag or separation of the tracer. The radioactive indicator does not alter progress of food as does barium(2), and unlike dye indicators it can be located accurately when mixed with feces. That progress of Sr^{89} was measured while absorption was occurring is an advantage in accord with the purpose for which the method was designed. Endogenous excretion of absorbed Sr^{89} was only 2% of administered dose in 6 hours, and only 6% dose in 24 hours(3). Krzywanek (4) observed that frequent feeding increased the rate of progress of barium meal through the intestinal tract. The present study shows that feeding also increased the movement of indicator from specific gut sites, when the indicator was administered some time after the food intake had ceased.

Advice of Dr. D. H. Copp is gratefully acknowledged.

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Received September 14, 1959. P.S.E.B.M., 1959, v102.

Plasmalogen in the Developing Brain.* (25300)

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Concentration of plasmalogen is quite different for different tissues(1,2). Furthermore, Yarbro and Anderson(3) showed that plasmalogen level in a tissue may change during rapid development or differentiation of tissue so that a "characteristic" plasmalogen level must be described not only in terms of specific tissue studied but also in terms of stage of development. Since brain tissue is known to change during early development as myelin is formed(4), we were interested in the relationship of plasmalogen concentration to stage of development of the brain. Analysis of rat brain showed that the plasmalogen level

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* This study was supported in part by Grant from Michigan Memorial-Phoenix Project, University of Michigan.

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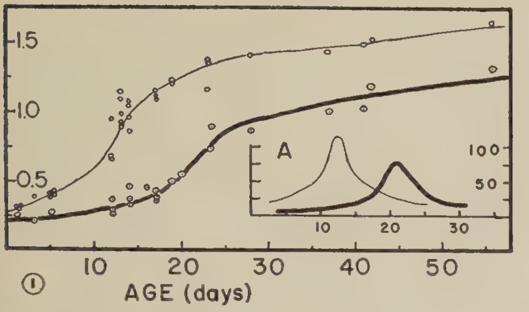

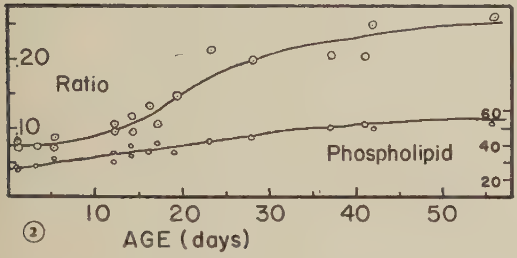

FIG. 1. Brain wt (thin line) is expressed as g of fresh tissue. Plasmalogen concentrations (heavy line) are in terms of μmoles of plasmalogen/100 mg of fresh tissue. Fig. 1A. Rate of change of brain wt (mg/day) is shown by the thin line and represents the slope of the brain wt curve. Increase in plasmalogen concentration (μmoles/g of tissue/day × 100) is shown by heavy line.

FIG. 2. Phospholipid concentrations are in terms of μmoles of phospholipid/g of fresh tissue. Ratio was calculated as μmoles of plasmalogen/μmole of total phospholipid.

is very low in 1-day-old rats but rises rapidly during second and third weeks of life. The high levels of plasmalogen, so far considered characteristic of brain, do not occur in the rat until the fourth week.

Methods. White rats (Sprague-Dawley strain), 1 to 56 days old were lightly anesthetized with ether and exsanguinated. Brains were removed, weighed, and stored at −20°C until lipid analyses were run. Lipid extract was prepared by homogenizing the frozen tissue in 4 ml of methanol, centrifuging, and treating precipitate with 3 ml of methanol. The remaining lipids were then extracted from the dehydrated tissue with two 5 ml portions of chloroform:methanol(2:1). All extracts were combined, 15 ml of chloroform were added, and the solution was washed with water(5). Phosphorus analyses were run by the Fiske and Subbarow(6) method. Plasmalogen analyses were run with Feulgen's reagents for aldehyde according to Rapport and Alonzo(7), and to avoid turbidity that occurred in the aqueous system, the colored pigment was extracted with 4 ml of isoamyl alcohol and read at 550 mμ.

Results in Fig. 1 show that the brain increased markedly in wet weight between 8 and 18 days, whereas plasmalogen concentration was relatively low for the first 2 weeks. If rate of change of brain weight is compared with that of plasmalogen concentration (as in Fig. 1a), the rate of accumulation of plasmalogen is optimal about 10 days after maximum rate of brain growth. Burton *et al.* (8) showed that enzyme activity for cerebroside synthesis varied with time and was maximal in 11- to 18-day-old animals. This observation agrees with the concept that cerebrosides are an important constituent of myelin and are formed at a high rate during times of myelin formation. In contrast, the results in Fig. 1 suggest that plasmalogen may not be involved in the earliest steps of myelin formation. Myelin is believed to be repeated layers of oriented lipid and protein derived from the Schwann cell membrane by simply an infolding process(9). If the plasmalogen in adult nervous tissues is located almost entirely in the myelin sheath as reported by Brante(10) and the major histological development of myelin in rat is between 10 and 20 days, then plasmalogen may be added to the Schwann cell membrane after its arrangement into the characteristic spiral formation around the axon.

During brain development the concentration of both plasmalogen and total phospholipid increased (Figs. 1 and 2). If these increases were due merely to formation of more cytoplasm and cellular membranes of the same type that existed in the newborn animal, one would expect the fraction of plasmalogen in the phospholipid to remain constant. This would be in keeping with the suggestion of Rapport and Lerner(11), who found that the plasmalogen-lipid phosphorus ratio for a given organ is relatively constant. These authors also showed that a variety of neoplastic tissues had a plasmalogen to phospholipid ratio comparable to that of corresponding normal tissue. On the other hand, there is no doubt that an organ can undergo a change in lipid composition if one type of structure within the organ develops to a disproportionate extent at some stage. Such a change occurs in the brain during myelination.

During the first 2 weeks accumulation of plasmalogen parallels that of other phospholipids so that the ratio (Fig. 2) is relatively constant (0.09 ± 0.02). This result agrees with the idea that more material of the same type is being formed. After 12 to 16 days, however, ratio increases, indicating a differential accumulation of plasmalogen. During the following 4 weeks the total phospholipids in the brain develop increasingly larger amounts of plasmalogen until a fairly constant composition (about 0.24) characteristic for adult mammalian brain tissue(1,2,11) is reached in the seventh week.

This shift in phospholipid composition to higher levels of plasmalogen does not occur in developing adipose tissue or liver. In these organs the per cent of plasmalogen in phospholipids actually decreases during the first 3 weeks(3). Although the plasmalogen-phospholipid ratio in brains of 1- to 14-day-old rats is similar to that found in a variety of other animal organs(11), ratios as high as those found in brain after 40 days have been found in only a few other sources of lipid, *viz.* ram spermatozoa(12), heart and skeletal muscle, and white matter(11). There is no apparent similarity in these sources that might relate plasmalogen content of an organ with function, but whatever the functional role of this phospholipid may be, plasmalogen becomes an increasingly significant constituent during the development of the brain.

Summary. Plasmalogen content in brains of newborn rats is about 2 μmoles/g of wet tissue and represents about 10% of total phospholipid. During growth, and after major increase in brain weight, phospholipid composition shifts to one containing about 20 to 25% plasmalogen (12 μmoles/g). Maximal rate of accumulation of plasmalogen in brain occurs in 20- to 24-day-old rats.

ADDENDUM: Somewhat similar results were reported by Korey and Orchen(13) as this manuscript was being prepared for publication.

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Received September 14, 1959. P.S.E.B.M., 1959, v102.

Progress and Rate of Absorption of Radiostrontium Through Intestinal Tracts of Rats.* (25301)

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In the following study a method was developed for estimating the effective absorption of radiostrontium from different parts of intestinal tract. This involves not only rate of absorption in each part, but also amount available for absorption. Since strontium is very similar to calcium in its behavior (despite biological discrimination factors) it is

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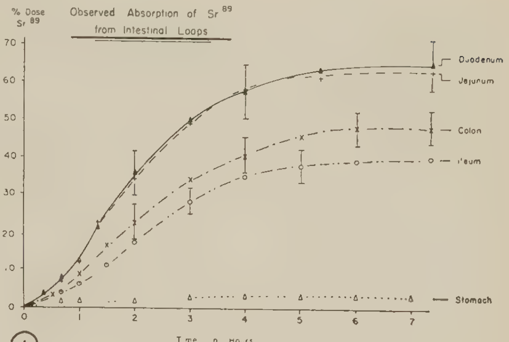
* Supported in part by extramural grant from Defense Research Board of Canada.

possible to use it as a qualitative indicator for the latter. The study also has some practical implications, since intestinal absorption is a principal route of entry of the hazardous isotope Sr^{90}. Bergeim(1) using inert indicators, found calcium absorption greatest in the small intestine of rats. Fournier(2) and Nicolaysen(3) found that calcium absorption rates were highest in first section of small intestine of rats. Moore and Tyler(4) obtained similar results in the pig. Jones and Coid(5), using ligated gut segments, found greatest absorption of Sr^{89}, during the first hour, in the duodenum. It was our purpose to evaluate the contribution of various parts of intestinal tract to strontium absorption. This was made possible by combining the use of 2 methods which measured rate of absorption and quantity of tracer continuously available for absorption in each division of the intestinal tract(6,7).

Methods. Adult female rats of Sprague-Dawley strain weighing 200-220 g were fasted overnight and anesthetized with nembutal-urethane mixture. The gut loop to be studied was tied above and below at any 2 of the following points; pylorus, duodeno-jejunal ligament, mid-point between this ligament and cecum, ileo-cecal juncture, and anal sphincter. Lengths of segments in centimeters averaged: duodenum 12, jejunum 42, ileum 42 and colón 14. Fifteen microcuries of carrier-free Sr^{89} in 1 ml saline were injected into the lumen of each loop. The fluid was injected slowly to avoid distention of gut. The rat was then placed in position such that radioactivity accumulating in the tail could be counted continuously, using a shielded Geiger counting tube, a ratemeter, and recording milliammeter. After 5 hours, little increase in tail radioactivity was observed. Rats were killed at 6 hours. Since the external tail count showed a constant ratio to the body content (6), the net absorption curve was derived from the curve of *in vivo* tail counts. The ratio was determined in each rat from final *in vivo* tail count and total carcass radioactivity after removal of gut contents. Passage of Sr^{89} along intestinal tract was measured by methods previously described(7).

Results. Fig. 1 shows *in vivo* absorption from each isolated gut segment. Each curve represents the average of 8 rats, all measured continuously. Standard errors are indicated by vertical lines. Absorption in the cecum is not shown in the Figure because it is almost negligible. The actual amount was 2.6 ± 0.2% dose in 24 hours.



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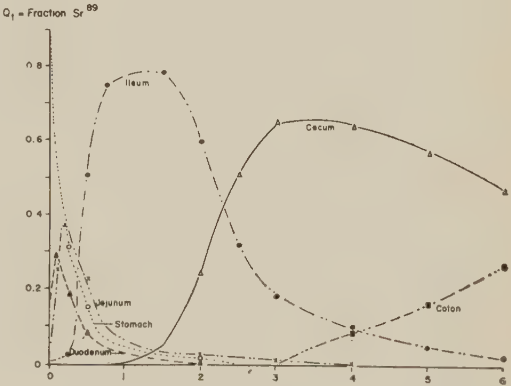

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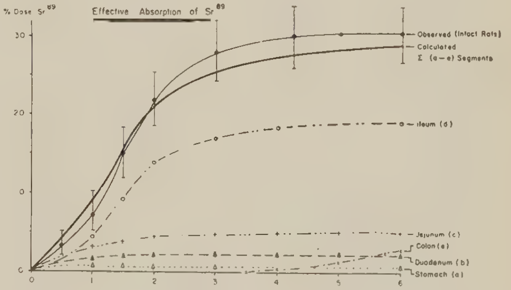

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FIG. 1. *In vivo* absorption of Sr^{89} from isolated segments of intestinal tract. Stand. errors are shown.

FIG. 2. Fraction of Sr^{89} in divisions of intact gut during transit. Each point represents an avg of values from at least 4 rats.

FIG. 3. Estimated effective absorption of Sr^{89} from divisions of intact gut.

Fig. 2 shows the fraction of dose of Sr^{89} found in main divisions of intestinal tract of intact rats at various times of sacrifice. Each value represents the mean of 5 animals. The differences of volume of different divisions and motility of the tract evidently determine the amount of isotope available for absorption in each segment.

The quantity of Sr^{89} available for absorption in each section (Fig. 2), can be combined with rate of absorption in that segment to estimate effective absorption. The steps are as follows: 1. Rate of absorption $\frac{dA}{dt}$ was taken as the tangent to the cumulative absorption curve (Fig. 1) of a given segment at any time t, in units of % dose/hour. 2. This rate was multiplied by $\frac{100}{100 - A_t}$ to obtain a rate in terms of amount of isotope present in the loop at time t. 3. This corrected rate is multiplied by quantity of isotope actually available for absorption (Q_t) in the segment to give the effective rate of absorption $\frac{dA_E}{dt}$. 4. Effective rates of absorption plotted against time were integrated graphically, to give cumulative effective absorption for each gut segment.

Fig. 3 shows the cumulative effective absorption in each segment, estimated as described above. This Figure also shows total effective absorption of all gut divisions added together (heavy line) compared to absorption in a separate group of intact rats given Sr^{89} by stomach tube. The calculations may be summarized by:

$$A_E = \int_0^t Q_t \left(\frac{dA}{dt} \times \frac{100}{100 - A_t} \right) dt \text{ where:}$$

A_E is net effective absorption over 6 hours.
Q is the fraction of Sr^{89} found in each gut segment of intact rats.
A is net absorption of Sr^{89}.

Discussion. Conflicting conclusions from previous studies of calcium sites of absorption may now be reassessed. Some methods of studying absorption measure only rate of absorption, and highest rates of absorption apparently occur in upper part of small intestine(2-5). High rates of absorption of Sr^{89} in upper half of small intestine are shown in Fig. 1. This confirms and extends a previous study of Jones and Coid, based on measurements covering the first hour(5).

In a ligated segment, all of administered material is available for absorption. Obviously all radioactive material in the intact animal is not confined to any one part of the gut. The actual amount of Sr^{89} available to each segment throughout the effective absorption period is shown in Fig. 2. As described above the 2 factors, *i.e.*, rate of absorption and availability of material, can be combined to estimate effective absorption in that segment in the intact animal. It is assumed that absorption rate in the ligated loop is not greatly altered from the rate in the same segment of intact animal for at least a few hours. It is significant that when the sum of effective absorption by all segments is plotted against time, the total compares very favorably with absorption curves for normal intact rats. This comparison is shown in Fig. 3. Further, if one considers size of various segments, their structure and motility, these values seem reasonable. Similar results were obtained with Sr^{85} when the experiment was repeated.

Effective absorption from the duodenum and jejunum was less than that of the ileum, principally because of their small content of Sr^{89} during period of effective absorption. The cecum and stomach were relatively ineffective due to a very low rate of absorption, and possibly prolonged storage of food in the cecum may be an important factor limiting absorption of strontium and related minerals. The part played by the colon is less easy to assess. The colon absorbed freshly administered Sr^{89} solution at a quite high rate, but little of the Sr^{89} which traveled through upper intestinal tract over 3 hours. The normal rat absorbs slowly after 3 hours. Observations indicate that more effective absorption occurs in the colon when conditions allow earlier filling of the colon. Since absorption was limited in time, both in the gut and in isolated loops, there appear to be factors in addition to movement of material through the gut which normally limit radiostrontium absorption.

Summary. Rate of absorption of Sr^{89} from ligated loops of rat intestine, and rate of passage of Sr^{89} through the intact intestinal tract were measured. Assuming only that absorption rate in ligated loops is reasonably the same as rate of absorption from that segment in the intact rat, the actual effective absorption was estimated. The highest initial rate of absorption occurred in the duodenum with jejunum, ileum, colon and stomach following in decreasing order. Since the radiostrontium passed rapidly through the duodenum and jejunum, the largest actual effective absorption occurred in the ileum (65%), with smaller contributions by the jejunum (17%), colon (8%), duodenum (7%), and stomach (2%). Two factors were found to limit Sr^{89} absorption: (1) Movement of the isotope into gut segments having slower absorption rate, and (2) decreased absorption of Sr^{89} in each gut loop with time.

The authors acknowledge the technical aid of Isabel Hewlett.

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Received September 14, 1959. P.S.E.B.M., 1959, v102.

Quantitative Measurement of Gastrointestinal Blood Loss During Ingestion of Aspirin. (25302)

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It has long been suspected that treatment with salicylates may induce gastrointestinal hemorrhage(1). Several recent studies(2-4) show that tests for occult blood in feces frequently become positive when salicylates are given. This report deals with the effect of ingestion of aspirin on gastrointestinal blood loss quantitatively assessed by measurement of fecal radioactivity in subjects whose erythrocytes were labeled with Cr^{51}.

Methods. Estimation of gastrointestinal blood loss with Cr^{51}. Twenty ml of venous blood was drawn and added to 10 ml of acid-citrate-dextrose solution containing 300 μ-curies of Cr^{51} as sodium chromate. The mixture remained at room temperature 30 minutes and then was injected intravenously. Starting 1 day later, oxalated blood samples were taken every 3 days. Serial 3-day composite stool collections in cardboard containers lined with bags of polyethylene film were begun 1 day after injection of tagged cells. Stool samples were weighed, a weighed amount of water was added, and homogenized in Waring blendor of 1 gallon capacity. Radioactivity of an aliquot of blood samples taken at beginning and end of each stool collection and of a weighed aliquot of homogenized stool was measured in a well-type scintillation detector with a gamma ray spectrometer set for Cr^{51} peak at 320 Mev. Gastrointestinal blood loss in ml/day was calculated as described by Ebaugh *et al.*(5). Subjects were adult male patients on gastroenterology wards of Wadsworth Veterans Hospital. Diagnoses, presence of upper gastrointestinal hemorrhage at time of entry to hospital, and incidence of history of gastrointestinal hemorrhage occurring while taking salicylate preparations are given in Table I. Patients who were bleeding when admitted to hospital were studied 2 or more weeks after evidences of hemorrhage had disappeared and, when study was begun, all were losing less than 2 ml of blood/day in the feces. *Administration of aspirin.* 28 subjects in Group 1 received 1 g of aspirin (3 tablets) 3 times a day

TABLE I. Diagnoses and Other Information on Subjects Studied.

Diagnosis	No. of subjects	No. bleeding on entry	No. with history of bleeding with salicylates
Group 1			
Duodenal ulcer	17	10	7
Gastric "	2		
Marginal "	1		1
Esophagitis	1	1	1
Cirrhosis	4	1	
Alcoholism	2		
Pancreatitis	1		
Group 2			
Duodenal ulcer	3	1	1
Pancreatitis	2		
Cirrhosis	5		

for 6 days. Stool and blood collections were made during, 3 days before, and 6 days after giving aspirin, a total of 5 3-day collections. Nine subjects in Group 1 received antacids (4 g CaCO$_3$ or 30 ml aluminum hydroxide gel) every waking hour during periods when aspirin was given. Group 2 of 10 subjects were studied for 7 three-day periods. During periods 2 and 5 they received 1 g aspirin 3 times a day, during 1 period as tablets, in other periods as a solution of 1 g aspirin powder plus 1 g NaHCO$_3$ dissolved in 100 ml water immediately before ingestion. Five subjects received tablets first and solution of aspirin second; the order was reversed in the other 5 subjects.

Results are summarized in Table II. Control levels of fecal blood loss ranged from 0.2 to 2.1 ml/day. In each subject fecal blood loss rose after aspirin was given, the peak rate occurring either during or in period immediately following administration of aspirin. For 38 subjects of Groups 1 and 2, the range of increases after aspirin (peak minus control) was from 0.2 to 13.5 ml/day, mean 3.3, stand. dev. 2.7. In 15 subjects the increase was less than 2 ml/day, in 16 between 2 and 5 ml/day, and in 7 it was 5 or more ml/day.

Analysis of variance showed that increase in fecal blood loss over the pre-drug level was statistically highly significant in both groups, for periods during and immediately following administration of aspirin (Group 1, $F = 13.95$, $P<0.005$; Group 2, $F = 4.86$, $P<0.01$). In Group 1 there were no significant differences between those who were bleeding when they entered hospital and those who were not, nor between those with history of bleeding associated with taking salicylates and those without such history. Patients who received antacids during periods when aspirin was given showed a smaller rise in fecal blood than those who did not receive antacids (period 3, $F = 7.07$, $P<0.01$; period 4, $F = 8.62$, $P<0.01$). In Group 2 fecal blood loss during period when aspirin was given in tablet form was not significantly different from that

TABLE II. Fecal Blood Loss before, during, and after Administration of Aspirin. (Means ± stand. dev. of fecal blood loss in ml/day.)

	No. of subjects	3-day period No. 1	2	3	4	5	6	7
GROUP 1								
		Treatment						
Subgroup		0	A	A	0	0		
All	28	1.1 ±.4	2.9 ± 2.4	4.0 ± 2.9	3.5 ± 1.9	2.1 ± 1.1		
Antacid	9	1.0	2.2	2.8	2.2	2.1		
No antacid	19	1.2	3.3	4.6*	4.1*	2.1		
Bleeding on entry	12	1.2	2.4	3.7	3.5	2.1		
No bleeding on entry	16	1.1	3.3	4.2	3.5	2.1		
Salicylate history	9	1.1	2.9	3.0	2.8	1.9		
No salicylate history	19	1.1	2.9	4.5	3.8	2.2		
GROUP 2								
		Treatment						
		0	SA	0	0	A	0	0
All	10	.9 ±.5	2.0 ± 1.5	1.4 ± 1.4	.9 ±.7	2.2 ± 1.6	2.2 ± 1.0	1.4 ±.4

Treatment: 0, no treatment; A, aspirin tablets, 1 g 3 times a day; SA, solution of aspirin, 1 g 3 times a day.

* Significantly different from comparison group.

during period when aspirin in solution was given ($F = 0.57$, $P>0.10$).

Discussion. Previous investigators(2-4), using qualitative tests for occult blood in feces concluded that gastrointestinal bleeding induced by aspirin occurred: a) with both soluble and insoluble aspirin, b) in subjects with or without demonstrable gastrointestinal lesions, and c) in subjects with or without history of hemorrhage while ingesting aspirin. These findings are substantiated by the present study with quantitative measurements of fecal blood loss.

In no instance could bleeding under these experimental conditions be classified as clinically important gastrointestinal hemorrhage. The factors involved in patients who experience major hemorrhage while ingesting aspirin await elucidation.

Summary. In humans whose erythrocytes were tagged with Cr^{51}, fecal excretion of the isotope was used to measure gastrointestinal blood loss. In 38 subjects, ingestion of 3 g of aspirin/day for 3 or 6 days increased fecal blood loss of 3.3 ml/day. Patients who had recently had upper gastrointestinal bleeding and patients who gave a history of gastrointestinal hemorrhage while taking salicylates did not differ in their response from subjects without such histories. Blood loss was smaller in subjects who received antacids every waking hour while taking aspirin than in those who did not receive antacids. Alkaline solution of aspirin produced the same effect as aspirin tablets.

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Received September 14, 1959. P.S.E.B.M., 1959, v102.

Photosensitization of Tissue Culture Cells and Its Effect on Viral Plaque Formation.* (25303)

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The phenomenon of photosensitization or photodynamic action of dyes and other chemical compounds is well known, and has been observed to affect various microorganisms and cells, as well as plants, animals and man(1). Stilwell described the photodynamic action of neutral red on cultures of embryonic chick cells(2), but no report has been found of photosensitization of agar-overlaid tissue cultures by the neutral red which is commonly contained in the overlay medium. On the other hand, neutral red itself has been reported to be lethal for some cell cultures, and to reduce viral plaque counts in others(3). During the course of experiments involving the use of rhesus monkey kidney culture cells for the study of viral plaques, prepared as originally described by Dulbecco(4) and modified by Hsiung and Melnick(5), it was noted that occasionally entire cell sheets or portions of sheets in some bottles inexplicably deteriorated. This deterioration was evidenced by a change in color from the usual pinkish-red to orange or yellow, and a homogeneous appearance of the cell sheet and agar overlay, similar to that seen in the confluent degeneration caused by infection with certain viruses, and generally interpreted as being indicative of death of the cells. Cultures exposed to direct light during incubation in a lighted "walk-in" incubator usually were the ones affected.

Methods. The hypothesis that photosensitization caused such destruction was studied by exposing cultures to a standardized source of light. A commercial bacteriological incu-

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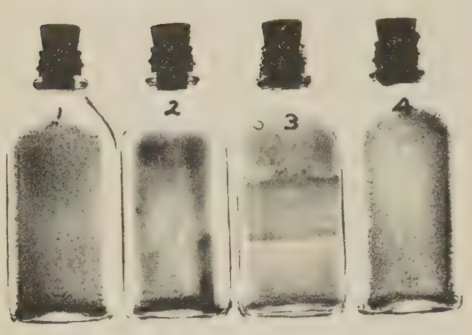
* Aided by grant from National Fn.



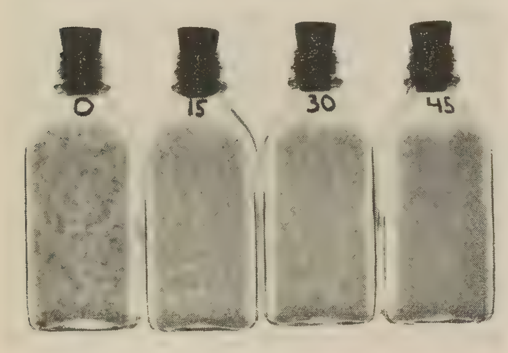


FIG. 1 (top). Effect of exposure to light on cultures of rhesus monkey kidney cells. Bottle 1, not exposed to light, shows intact sheet of cells; in bottle 2, exposed to light in presence of neutral red, no cell sheet is visible; bottle 3, treated in same manner as #2 except for application of a strip of masking tape during period of light exposure, shows only a band of the cell sheet remaining in area shaded from light; bottle 4, exposed to light in absence of neutral red shows a good cell sheet indistinguishable from that of bottle 1.

FIG. 2 (bottom). Effect of exposure to light on plaque formation of type 1 poliovirus in rhesus monkey kidney cells. Photograph was taken 48 hr after each culture had been inoculated with 100 plaque forming units of virus. Bottle 0 was not exposed to light; the others were exposed to light 15, 30, and 45 min. as designated. Note apparent reduction in size and No. of plaques which decrease progressively with increases in time of exposure.

bator, measuring 20 × 22 × 30 inches on the inside, was equipped with a fluorescent light fixture consisting of two, 15-watt, white units, attached to the ceiling. Bottles containing cultures were exposed to direct light by placing them in a tray on a shelf 20 inches beneath the light source. One half of the tray was covered by a piece of plywood, and cultures placed in the covered half of the tray were thus protected from exposure to direct light and served as controls. Most experiments employed rhesus monkey kidney cells grown in 3-ounce prescription bottles, and overlaid with agar containing neutral red in a final concentration of 1:60,000, in accordance with a commonly employed procedure of plaque assay(5). In order to determine that the degenerative changes observed were due to the photodynamic effects of neutral red, some cultures were overlaid before exposure to light in exactly the same manner except that the overlay medium contained no neutral red. A typical experiment consisted of preparing 8 bottle cultures, 4 with an overlay medium containing neutral red and 4 with the same medium without neutral red. As soon as the agar overlay had solidified, 4 bottles—2 with and 2 without neutral red—were exposed to light, while 4 similar bottles were placed in the same tray but shaded from light, in the manner described above. After exposure, cultures without neutral red in the first overlay received a second overlay containing neutral red, whereas those with neutral red in the first overlay received a second overlay without neutral red. All cultures were then incubated in the dark for a day or two.

Results. The cell sheets of cultures which had not been exposed to light had the usual fine lacy, pinkish-red appearance; moreover, those which had been exposed to light in the absence of neutral red presented a similar appearance, while the cell sheets of cultures exposed to light in the presence of neutral red actually could not be recognized, and the contents of bottles containing such cultures appeared homogenous and pinkish-yellow or orange. Exposure for as little as 2 or 3 hours resulted in visible degenerative changes. Furthermore, cultures which had developed the typical pinkish-red appearance after application of the usual overlay containing neutral red and incubation in the dark, when subsequently exposed to light, underwent similar deterioration. The photosensitizing action of neutral red was demonstrable with both fluorescent and incandescent light; and tissue cultures of Hep 2, chick embryo kidney, and dog kidney, also were found to be susceptible. Fig. 1 demonstrates the effects of exposure to light on photosensitized monkey kidney cells.

The possible influence of photosensitivity on viral plaque formation was also studied.

Titrations, in quadruplicate, of type 1 poliovirus (Mahoney strain) were carried out, by the plaque-assay technic, in bottle cultures of rhesus monkey kidney cells. Cultures inoculated with various amounts of virus were exposed to light for varying periods of time, and compared with control cultures which had not been exposed to light. Periods of exposure insufficient to produce discernible changes in the cell sheets were chosen. After 48 hours of incubation in the dark, following infection, plaques were often found to be smaller and less numerous in the cultures exposed to light than in unexposed controls. On further incubation, however, these differences were minimized or obviated. Fig. 2 illustrates the effect of light on the plaque formation of type 1 poliovirus in tissue cultures.

Summary. Degenerative changes occur in agar-overlaid tissue culture cells which have been exposed to light in the presence of neutral red. Temporary modification of the ability to form poliovirus plaques may occur in such cells when exposure to light is insufficient to produce discernible changes in the cell sheet.

Addendum. After this paper had been submitted for publication a report (Klein, S. W., Goodgal, S. H., *Science,* 1959, v130, 629) appeared which contained data similar to that presented above.

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Received September 15, 1959. P.S.E.B.M., 1959, v102.

Action of Testosterone in Blocking Urinary Electrolyte Effects of Desoxycorticosterone.* (25304)

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Two distinct types of steroids were reported recently to antagonize renal electrolyte effects of mineralocorticoids. The steroidal 17-spirolactones(1) were discussed as first synthetic examples with aldosterone- and desoxycorticosterone-blocking activity in the laboratory (2-4) and in man(4,5). By virtue of electrolyte effects which were dependent upon 1) presence of mineralocorticoids and 2) dosage ratio of blocking agents/mineralocorticoids, the mechanism of action of spirolactones was ascribed to competitive inhibition. A naturally occurring steroid, progesterone, was also reported to possess a similar blocking ability(6-9), but it demonstrated desoxycorticosterone-like effects on electrolyte excretion when given alone at large doses(6). The present report summarizes ability of testosterone, another gonadal hormone, to reverse electrolyte effects of desoxycorticosterone in rats.

Method. Sprague-Dawley male rats (150 to 200 g) were adrenalectomized under ether anesthesia, then maintained on 0.85% sodium chloride solution and Purina Chow *ad lib.* Approximately 6 days postoperatively animals were injected subcutaneously with 2.5 ml isotonic saline and with 12 μg of desoxycorticosterone acetate (DCA) in 0.1 ml Mazola oil. Groups were injected additionally with 0, 2, 4, 8 and 20 mg of testosterone in 0.5 ml for study of blocking effects. Other animals received corresponding doses of testosterone without DCA. Animals were placed in metabolism cages (5 rats/cage) for collection of 4-hour sample of urine. Pooled urine from each cage was diluted with water and analyzed for total Na and K content using Beckman flame photometer. Values for each sample were used directly for computation of the Na/K ratio. DCA alone reduces the ratio of

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* The authors acknowledge technical assistance of Janet L. Wallen and Clarice A. Kieffer.

TABLE I. Effects of Testosterone Given Alone and in Combination with DCA on Urinary Na, K and Na/K in Adrenalectomized Rats.

Treatment/rat		Mean urinary values		
DCA, μg	Testosterone, mg	Na	K	Na/K
12	—	1.34	1.32	.91
”	2	1.57	1.27	1.24*
”	4	1.35	.98*	1.30*
”	8	1.70	1.03*	1.64*
”	20	1.84*	.97*	1.93*
—	—	2.10	1.20	1.76
—	2	1.95	1.14	1.72
—	4	1.57*	.92*	1.72
—	8	1.66	.97	1.75
—	20	1.69	.95*	1.79

* $P < .05$, relative to DCA or untreated controls; least significant difference ($P = .05$) = ± .45 for Na, ± .25 for K and ± .27 for Na/K; Na and K values = mM output/4 hr/5 rats; 4 measurements for each test response and 8 for DCA and untreated controls.

Na/K in the test by causing Na retention and K loss. Ability of testosterone to block DCA was assessed by reversal of the Na/K response. Progesterone was also tested conjointly in this study, alone and in combination with DCA, for comparative data. The pooled standard deviation (s) of various groups was 0.217 for Na/K; thus, the least significant difference ($P = 0.05$) between controls ($N = 8$) and treated ($N = 4$) groups was ± 0.27 ($t = 2.0$ at 62 degrees of freedom). The pooled s for Na and K response was 0.371 and 0.205, and thus, the least changes for significance were ± 0.45 and ± 0.25, respectively.

Results. The action of testosterone in blocking electrolyte effects of DCA is illustrated in Table I. DCA alone at the standard dose of 12 μg/rat produced a low value of 0.91 for Na/K (*cf.* Na/K = 1.76, for untreated controls). Testosterone significantly blocked DCA at a dose of 2 mg; larger doses progressively reversed the Na/K response to that found in untreated controls. In general, blocking resulted from simultaneous antagonism of Na retention and K loss. The Table also shows that testosterone alone was without effect on Na/K, with responses at 2 to 20 mg approximating closely the value obtained for untreated controls. There was at the same time a tendency for a slight but definite degree of Na and K retention at 4 and 20 mg levels.

Fig. 1 demonstrates the dose-response curves with testosterone and progesterone in terms of Na/K effect of DCA. The 2 steroids were approximately equipotent in reversing the Na/K response at 2 and 4 mg doses. An increasing dose-response curve was obtained with testosterone (slope = 0.72/10-fold range of log doses). With progesterone, the strongest reversal was achieved with the 4 mg dose; increasing doses produced, as a contrast, smaller degrees of blocking effects. The Na/K responses following treatment with the 2 steroids were significantly different at 8 and 20 mg dosage levels ($P < 0.05$). Reduction of blocking potency with progesterone appears to have resulted primarily from its DCA-like effects on Na/K (Table II).

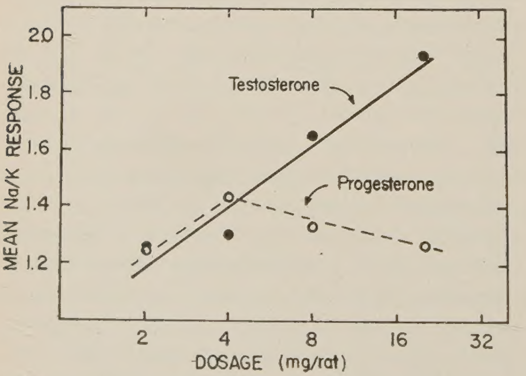

FIG. 1. Dose-response curves demonstrating action of testosterone and progesterone in blocking effects of 12 μg of DCA on urinary ratio of Na/K in adrenalectomized rats. All responses are significant ($P < .05$), relative to DCA controls.

Discussion. Competitive inhibition would be suggested as the mechanism of DCA-blocking effect for testosterone if the Na/K data are used for interpretation. Increasing doses, *e.g.*, cause greater blocking of DCA to suggest importance of dosage ratio of testoster-

TABLE II. Effects of Progesterone Alone on Urinary Ratio of Na/K in Adrenalectomized Rats.

Treatment/rat, progesterone, mg	No. of measurements	Mean ratio Na/K
—	8	1.76
2	4	1.56
4	”	1.43*
8	”	1.46*
20	”	1.16*

* $P < .05$, relative to untreated controls; least significant difference for P of .05 = ± .27; 5 animals/measurement.

one/DCA. When given alone, the steroid did not affect Na/K in a manner opposing DCA action, to rule out physiological antagonism as the possible mechanism of effect. Thus, it appears that the compound exerts electrolyte effects by competing with DCA for receptor sites in target tissues, as described for other blockers(2,3,6). Inhibition is believed to be at the renal level, the primary site of action of mineralocorticoids for effects on electrolyte excretion(10-12). Unlike progesterone, testosterone did not possess DCA-like effects on Na/K when given alone; it produced for this reason an increasing blocking response over a relatively wide range of doses. The steroid appeared to be as potent as progesterone for minimal blockade; at large doses, it gave significantly greater blocking effects than progesterone. If the relative potencies in our study extrapolate to man, it would appear that testosterone would be at least as effective as progesterone in antagonizing mineralocorticoids in Addisonians(7-9). The steroid as produced endogenously under normal conditions would not be expected to influence electrolyte metabolism if body fluid titers of the hormone are gauged by doses required for replacement therapy in hypogonadal states (13-15). Potentially, however, it may influence electrolyte excretion by representing one member in a pool of structurally related blocking agents.

Summary. Large doses of testosterone blocked urinary electrolyte effects of desoxycorticosterone acetate (DCA) in adrenalectomized rats. The steroid reversed the Na/K response to DCA by simultaneously blocking Na retention and K loss. It did not affect Na/K in a manner opposing DCA action when given alone. These observations suggest competitive inhibition as the mechanism of effect for testosterone. Unlike progesterone, it was devoid of DCA-like effects on Na/K given alone, and it gave increasing DCA-blocking effect over a relatively wide range of doses.

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Received September 21, 1959. P.S.E.B.M., 1959, v102.

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CONTRIBUTION received from Johnson and Johnson